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13. ABSTRACT (Maximum 200 Words)

Osteocalcin (OC) expresses in both normal and cancerous prostate epithelial cells and prostate cancer metastasis to the bone and lymph node. The goal of this research is to develop a novel therapeutic agent(s) based on osteocalcin promoter for the treatment of prostate cancer, particularly bone metastasis. Three therapeutic agents were proposed for testing: thymidine kinase (TK), cytosine deaminase (CD), and Diphtheria toxin-interleukin 13 fusion toxin (DT-IL13). Recombinant adenovirus, Ad-OC-TK, carrying TK under the control of mouse OC promoter, with prodrug, has therapeutic efficacy on hormone refractory prostate cancers in animal models and had no toxic side effects in our formal toxicology study. The result of this study has been translated to a phase I clinical trial which will be reported publicly the near future. Ad-OC-CD did not demonstrate activity in killing prostate cancer cells when supplemented with prodrug 5-FC. DT/IL13 was demonstrated to be toxic when expressed in the 293 cell, so DT/IL13 study was terminated. Due to the weak and non-specific activity of OC promoter identified in this study, we investigated a hybrid prostate-specific promoter, called PSES, from PSA and PSMA genes. PSES demonstrated very strong prostate-specific activity. Its use in prostate cancer gene therapy is currently under investigation.

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prostate cancer, osteocalcin, gene therapy, metastasis, adenovirus

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INTRODUCTION

Metastatic human prostate cancer (PC) is commonly treated by hormone, radiation, and/or chemotherapy. Inevitably, these patients will eventually relapse and develop androgen-independent disease with osseous metastasis. Since no effective therapy is presently available for the treatment of PC metastasis, we are developing a novel gene therapy modality for hormonal refractory prostate cancer based on tissue-specific osteocalcin (OC) promoter. OC expression was found in most of the micro-calcified tumors and localized and metastatic PC. In this study, we proposed to compare the therapeutic efficacies of thymidine kinase (TK), cytosine deaminase (CD) and bacteria Diphtheria toxin-interleukin 13 fusion (DT-IL13) genes under the control of mouse OC promoter in PC experimental models. Three Specific Aims were proposed: **1)** To determine the expression of OC and PSA, a prostate specific antigen, in specimens of both localized and metastasized PC. The expression of OC mRNA in tumor specimens was studied with in situ hybridization. **2)** To construct and compare the effectiveness of three types of therapeutic genes in adenoviruses (Ad): Ad-OC-TK, Ad-OC-CD and Ad-OC-DT-IL13, in killing PC cells. **3)** To determine the distribution and activity of these viruses in the host vital organs including the bone and in PC xenografts to ascertain the toxicity and therapeutic efficacy of these viruses.

BODY

Technical Objectives A:

Task 1:   PSA and OC immunohistochemical staining study.

Our initial immunohistochemical staining study showed that one hundred percent of primary (3/3) and metastatic prostate cancers such as lymph node (8/8) and osseous metastases (10/10) prevalently expressed osteocalcin. Another postdoctoral fellow could not reproduce the original observation. Instead, he found 2/15 primary prostate cancers stained positive for OC; 4/13 lymph node metastases stained positive for OC; 9/14 bone metastases stained positive for OC (see Table 1).

Task 2:   in situ hybridization study.

In contrast to immunohistochemical staining, in situ hybridization study revealed 100% of prostate cancers, 15 primary cancer, 13 lymph node metastases and 14 bone metastases express OC mRNA (Table 1).

Task 3:   purify RNA from tumor cells, Northern blot analysis.

Multiple human tissue Northern blots were ordered from Clontech (Palo Alto, CA). These Northern blots were hybridized with ^{32}P-labeled OC cDNA probe according to the manufacture's protocol. The result revealed two transcripts which hybridized to OC cDNA probe. Bone marrow and heart had strong expression of the low molecular weight transcript with the molecular weight of reported OC mRNA. Luo et al reported that OC played an important role in the inhibition of artery calcification. The expression of OC in the heart might be contributed by artery tissue. Besides bone marrow and heart, several other organs, such as trachea, liver, and skeleton muscle also expressed a low amount of low molecular weight OC mRNA. Thyroid, prostate, and ovary had moderate expression of the high molecular weight transcript. PCR analysis of RNA extracted from human prostate cancer cell line also revealed two products. Sequencing analysis of those PCR products revealed that the low molecular weight product made from the reported OC mRNA and the high molecular weight product contained intron sequences of OC gene and was likely a splicing variant. The result is consistent with

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immunohistochemical staining and in situ hybridization, and suggests that normal prostate epithelial cells make OC transcript but do not splice the RNA properly. When prostate cancer cells metastasize to the bone, they learn the way to splice OC transcript properly and make OC proteins. This study was published in Gene (see attached paper 1).

Table 1:
0, no reactivity
1, localized signal and low reactivity
2, diffuse and low to moderate reactivity
3, diffuse and high reactivity

Sample	Tissue	ISH	IHC	Remarks
1	prostate	3	1	biopsy
2	prostate	3	0	biopsy
3	prostate	2	0	biopsy
4	prostate	2	0	biopsy
5	prostate	3	0	biopsy
6	prostate	3	0	biopsy
7	prostate	2	0	biopsy
8	prostate	3	0	biopsy
9	prostate	3	0	biopsy
10	prostate	3	1	autopsy
11	prostate	3	0	autopsy
12	prostate	3	0	autopsy
13	prostate	3	0	autopsy
14	prostate	3	0	autopsy
15	prostate	3	0	autopsy
16	LN	2	0	biopsy
17	LN	2	2	biopsy
18	LN	3	0	biopsy
19	LN	2	0	biopsy
20	LN	3	1	biopsy
21	LN	3	0	biopsy
22	LN	3	0	biopsy
23	LN	2	0	biopsy
24	LN	2	0	biopsy
25	LN	3	0	biopsy
26	LN	2	1	biopsy
27	LN	3	0	autopsy
28	LN	3	0	autopsy
29	bone	1	0	biopsy
30	bone	3	1	biopsy
31	bone	1	0	biopsy
32	bone	2	0	biopsy
33	bone	3	1	biopsy
34	bone	2	1	biopsy
35	bone	2	1	biopsy
36	bone	3	1	biopsy
37	bone	2	0	biopsy
38	bone	3	0	biopsy
39	bone	3	3	autopsy
40	bone	3	3	autopsy
41	bone	3	3	autopsy
42	bone	3	3	autopsy

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Technical Objectives B:

Task 4: construct shuttle vector pΔE1sp1A-OC-CD and pΔE1sp1A-OC-DT-IL13; large scale purification of pΔE1sp1A-OC-CD and pΔE1sp1A-OC-DT-IL13; determine if 293 cells may contain OC-promoter activity and IL13 receptor.

Construction of pΔE1sp1A-OC-CD was finished. Construction of pΔE1sp1A-OC-DT-IL13 was abandoned due to nonspecific toxicity of OC-DT-IL-13.

Task 5: generate Ad-OC-CD and Ad-OC-DT-IL13 by homologous recombination between pJM17 and Ad-OC-CD or Ad-OC-DT-IL13, respectively; plaque purification of Ad-OC-CD and Ad-OC-DT-IL13.

Ad-OC-CD was constructed and purified. Construction of Ad-OC-DT-IL13 is abandoned as described in Task 4.

Task 6: Ad-OC-CD/5-FC and Ad-OC-DT-IL13 in vitro cell killing assay.

Ad-OC-CD/5-FC in vitro cell killing assay was performed. Ad-OC-CD/5-FC did not have cell killing activity on prostate cancer cells. This might result from low mouse OC promoter activity in prostate cancer cells. In vitro cell killing activity test of Ad-OC-DT-IL13 is abandoned as described in Task 4.

Task 7: large scale preparation of Ad-OC-TK; analyze virus titer and activity.

Finished.

Task 8: first therapeutic efficacy study on Ad-OC-TK/ACV.

Ad-OC-TK/ACV was able to inhibit prostate cancer, C4-2 and PC-3, growth in nude mice. This result prompted us to conduct a phase I clinical trial (see attached paper).

Task 9: small scale preparation of Ad-OC-CD and Ad-OC-DT-IL13; analyze virus titer and activity.

Small scale preparation of Ad-OC-CD was finished. The preparation of Ad-OC-DT-IL13 is abandoned as described in task 4.

Task 10: first therapeutic efficacy study on Ad-OC-CD/5-FC.

Ad-OC-CD/5-FC did not have killing activity against prostate cancer cells, LNCaP and PC-3.

Task 11: first therapeutic efficacy study on Ad-OC-DT-IL13.

This study is abandoned as described in task 4.

Task 12: Months 22-26: second therapeutic efficacy study on Ad-OC-TK/ACV, Ad-OC-CD/5-FC and Ad-OC-DT-IL13

This study is not conducted due to the lack of therapeutic activity of Ad-OC-CD/5-FC and the toxicity of OC-DT-IL13.

Technical Objectives C:

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Task 13: small scale preparation of Ad-CMV-ß gal and Ad-OC-ß gal; analyze virus titer and activity.

Finished.

Task 14: establish PC-3 tumors; intratumor injection of Ad-CMV-ß gal and Ad-OC-ß gal; harvest organs.

Finished.

Task 15: ß gal immunohistostaining; ß gal enzyme assay; RNA extraction and RT-PCR analysis; DNA extraction and PCR analysis.

Ad-OC-ß gal has very low activity in PC-3 cells.

Task 16: toxicity study on Ad-OC-TK/ACV.

Finished. In order to facilitate the Ad-OC-TK gene therapy clinical trial for prostate cancer we decided to perform the formal toxicology study at University of Pennsylvania. Intraosseous administration of Ad-OC-TK supplemented with intraperitoneal injection of prodrug Valtrex did not induce any significant toxicity at all levels of examination (see appendix 3: a summary report from the University of Pennsylvania. Ad-OC-TK was named as H5-OC-TK in the report). This result has been included in the IND application and a Phase I clinical trial has been initiated (see attached paper 2).

Task 17: large scale preparation of Ad-OC-CD or Ad-OC-DT-IL13; analyze virus titer and activity.

This study is not conducted due to the lack of therapeutic activity of Ad-OC-CD/5-FC and the toxicity of OC-DT-IL13.

Task 18: Months 14-17: toxicity study on Ad-OC-CD/5-FC.

Same as Task 17.

Task 19: Months 27-30: toxicity study on Ad-OC-DT-IL13.

Same as Task 17.

Due to the disappointing results from the study of OC expression pattern in human tissues, lack of therapeutic efficacy of Ad-OC-CD/5-FC, and toxic effect of OC-IL13, we decided to change our research direction. Instead of using OC promoter, we generated a novel prostate specific chimeric promoter, PSES, from PSA and PSMA enhancer elements. PSES demonstrated strong prostate specific activity in the absence of androgen (see attached paper 4). We are currently investigate the use of PSES to construct a prostate-specific replication competent adenovirus to treat androgen-independent prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrate prevalent OC mRNA expression in prostate cancer specimens, including primary tumor and lymph node and osseous metastasis. This lays the base for the use of Ad-OC-TK/Valtrex gene therapy to treat prostate cancer.

2. Demonstrate the safety of Ad-OC-TK/Valtrex gene therapy and promote Ad-OC-TK/Valtrex into a Phase I clinical trial. However, Ad-OC-TK/Valtrex gene therapy clinical trial did not demonstrated significant therapeutic effect.

3. Detect OC transcripts in several other organs. This result revealed, the first time, that OC expression was control at 2 levels in human. The first level of control is on transcription; the second level of control is on RNA splicing. Proper splicing of OC RNA only occurs efficientlyt in osteoblast.

4. We have generated a novel prostate specific promoter, PSES, which is highly active in androgen-independent prostate cancers. PSES is a hybrid from PSA and PSMA enhancers. Since the majority of androgen-independent prostate cancer patients express PSA and PSMA, PSES is expected to exhibit high activity in androgen-independent prostate cancers. We are currently constructing a prostate-restricted replication competent adenovirus based on PSES chimeric promoter. We believe that this PSES-based prostate-restricted replication competent adenovirus will be in clinical trial in the near future.

REPORTABLE OUTCOMES

The result of this study was presented in the 8th Prouts Neck Prostate Cancer Conference on October 21-24, 1999, and published in 3 papers.

1. Koeneman KS, **Kao C**, Ko SC, Yang L, Wada Y, Kallmes DF, Gillenwater JY, Zhau HE, Chung LWK, Gardner TA. Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. World J. Urol. 18:102-110, 2000.

2. Jung C, Ou Y-C, Yeung F, Frierson, HF Jr, **Kao C.** Osteocalcin is incompletely spliced in non-osseous tissues. Gene 271:143-150, 2001.

3. Lee S-J, Kim H-S, Yu R, Gardner TA, Jung C, Jeng M-H, Yeung F, Cheng L, **Kao C.** Novel prostate-specific promoter derived from PSA and PSMA enhancers. Mol. Therapy 6:415-421, 2002.

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CONCLUSIONS

This study demonstrated that human prostate cancers, including primary tumors and metastases, expressed OC mRNA. These encouraging observations suggested that the OC promoter was an excellent tissue-specific candidate promoter for the delivery and expression of therapeutic toxic genes in primary and metastatic human prostate cancers.

The toxicology study demonstrated the safety of Ad-OC-TK/Valtrex gene therapy for the treatment of prostate cancer osseous metastases. This result prompted us to conduct a Phase I clinical trial using Ad-OC-TK/Valtrex gene therapy for the treatment of prostate cancer. We treated patients with Ad-OC-TK/Valtrex. The result demonstrated the safety of Ad-OC-TK/Valtrex; however, therapeutic efficacy was limited. We believed that the limited responses to the therapy was due to the following reasons. First, gene delivery efficacy in vivo is low. Second, TK/ Valtrex only functions well in quick growing cell. The majority of prostate cancers are slow growing tumors, so TK/ Valtrex is not a good candidate for prostate cancers.

Recombinant adenovirus, Ad-OC-CD, carrying CD gene under control of mouse OC promoter did not have therapeutic efficacy on human prostate cancer cells. DT-IL13 was toxic when expressed, so OC-DT-IL13 was toxic to all cell lines tested without any tissue specificity.

Northern blot analysis revealed two OC mRNAs. Heart and bone had the strongest expression of OC mRNA (low molecular weight) in all tissues tested. Our toxicology study did not detect any adverse effect on the heart. This might due to the low infectivity of heart cells in our study conditions and little or no toxicity of TK/Valtrex to non-dividing cells such as heart or artery cells. Moderate expression of OC mRNAs was also detected in several other tissues including liver, skeletal muscle, ovary, thyroid, trachea, and prostate. Prostate cell expressed high molecular weight OC mRNA. PCR analysis of RNA from prostate cancer cell lines revealed several products. Sequencing analysis of the PCR products revealed that the high molecular weight products contained intron sequence of OC gene. The two OC mRNAs expressed by different organs are likely to be derived from a different splicing event. This result suggests that more studies are required to support the use of OC promoter-based gene therapy in the future.

To continue our effort in developing novel therapeutic agents for androgen-independent prostate cancers, we explored 2 other prostate specific promoters, PSA and PSMA. The main prostate-specific activity of PSA promoter is located in the upstream enhancer core, AREc, and its activity relies on androgen. On the other hand, the main prostate-specific activity of PSMA promoter resides in an enhancer core, PSME, in the third intron of the PSMA gene. Its activity is suppressed by androgen. Both AREc and PSME apparently function weakly in patients receiving androgen-ablation therapy due to the presence of low levels of androgen in the patients and AR mutation or amplification that will partially activate AREc and suppress PSME activity. In a serial study, by detecting and deleting silencers, we generated stronger enhancer cores from AREc and PSME, called AREc3 and PSME(del2), respectively. A combination of AREc3 and PSME(del2), called PSES, demonstrated strong synergistic and prostate-specific activity regardless of androgen status. We are currently developing a PSES-based prostate-specific replication-competent adenovirus for androgen-independent prostate cancers.

REFERENCES

APPENDICES

1. Jung C, Ou Y-C, Yeung F, Frierson, HF Jr, **Kao C.** Osteocalcin is incompletely spliced in non-osseous tissues. Gene 271:143-150, 2001.
2. Koeneman KS, **Kao C**, Ko SC, Yang L, Wada Y, Kallmes DF, Gillenwater JY, Zhau HE, Chung LWK, Gardner TA. Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. World J. Urol. 18:102-110, 2000.
3. Toxicology study report.
4. Lee S-J, Kim H-S, Yu R, Gardner TA, Jung C, Jeng M-H, Yeung F, Cheng L, **Kao C.** Novel prostate-specific promoter derived from PSA and PSMA enhancers. Mol. Therapy 6:415-421, 2002.

Osteocalcin is incompletely spliced in non-osseous tissues

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Abstract

Osteocalcin (OC) is known to be a bone tissue-specific protein, expression of which is believed to be controlled by the OC promoter. In this communication, we provided evidence to demonstrate that tissue-specific expression of OC was also regulated at the RNA splicing level. We identified incompletely spliced variants of human OC mRNA, which retain one or more introns during RNA spicing, existing dominantly in non-osseous organs. Northern blot analysis identified two OC RNA transcripts expressed in normal human tissues, but the expression level of the transcripts varied between the tissues. Most non-osseous tissues expressed transcripts with higher molecular weight, prominent in ovary, kidney, pancreas, spleen, thymus, prostate, and testis, than the expected size of OC mRNA as seen in bone marrow. RT-PCR analysis identified up to six OC transcripts in most tissues tested except bone marrow. Sequence analysis showed that four of five RNA variants contained intron 1 in common and the dominant one contained all three introns. MG63, an osteoblastic osteosarcoma cell, expressed only the completely-spliced form of OC, whereas incompletely spliced RNA was dominant in most prostate tumor cells. Combined study of *in situ* hybridization and immunohistochemistry revealed that OC RNA was highly expressed in prostate tumor epithelial cells while only very low levels of protein were detected, which confirms that there are OC RNA variants in non-osseous tissues. In conclusion, we demonstrated that OC mRNA is also expressed in several non-osseous tissues. However, only bone preferentially underwent the complete splicing event of all three introns. The function of other splicing variants of OC mRNA needs to be further investigated. © 2001 Published by Elsevier Science B.V. All rights reserved.

Keywords: Splicing; Bone; Intron retention; RNA transcript

1. Introduction

Osteocalcin (OC) is a member of a larger family of proteins, called Gla proteins since OC contains three glutamic acid residues (Gla residues) that are γ-carboxylated in a vitamin K-dependent manner (Poser et al., 1980). There are two OC-related genes, OG1 and OG2, in mice, which are expressed only in bone, whereas the other OC-related gene (ORG) is transcribed in kidney (Desbois et al., 1994). Although a human OC gene cluster is not yet known, human OC is composed of four exons and three introns and codes for 100 amino acid OC precursor, which cleaved to produce a mature peptide of 51 amino acids (Celeste et al., 1986; Kiefer et al., 1990).

OC constitutes 1–2% of the total protein in bone and its expression is restricted in osteoblasts. OC functions at later stages of bone formation in the regulation of mineral deposition and turnover of bone. Recently, knockout mice studies demonstrated that OC would be a negative regulator of bone formation by limiting bone matrix formation and inhibiting calcification (Ducy et al., 1996; Luo et al., 1997). A number of studies also reported that OC transcription is enhanced in mature osteoblasts while suppressed in early progenitors (Desbois et al., 1995; Boskey et al., 1998). Other than bone-related tissues, OC is synthesized by vascular smooth muscle cells (Watson et al., 1994) and its mRNA is also expressed in megakaryocytes and peripheral blood platelets in rat, which possibly contribute to the OC levels in blood and the regulation of bone turnover (Thiede et al., 1994).

The regulation of the OC gene is very complex. Most studies have emphasized transcriptional regulatory mechanisms related to OC promoter and its transcriptional factors that control suppression of the OC gene in bone progenitor cells and transactivation in mature osteoblasts (Lian et al., 1998). In this study, we describe the presence of variant OC

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mRNA isoforms from normal human tissues except osteo-blast-related cells, which possibly encode a subtype of OC protein. Selected human tumor cell lines were also studied for differential RNA splicing. To see the differential expression and tissue distribution of OC RNA and protein, combined assay of *in situ* hybridization (ISH) and immuno-histochemistry (IHC) was performed in human prostate tumors.

2. Materials and methods

2.1. Cell lines and tissues

MG63, human osteosarcoma cell lines, and four human prostate cancer cell lines, LNCaP, C4-2, PC3, and Du145, were studied. Cells were routinely cultured in T-medium (Life Technologies, Grand Island, NY) supplemented with 5% FBS at 37°C in an atmosphere containing 5% CO_2 as described previously (Wu et al., 1998). All cultures were fed with fresh medium every 3–4 days. Cells were grown until 80–90% confluent on P100 culture dishes before total RNA was extracted. For ISH study, all formalin-fixed and paraffin-embedded tissues were obtained from the Pathology Department of the University of Virginia.

2.2. Oligonucleotides

For RT-PCR analysis, two sets of primers were generated from the reported nucleotide sequence of the human OC gene (Celeste et al., 1986). A set of primers, OC1FW and OC1RV (corresponding to nucleotides 509–528 and 1355–1374, respectively), in the human OC gene produces the designated 238 bp PCR product specific with OC cDNA from osteoblasts. Another set of primers, OC2FW and OC2RV (corresponding to nucleotides 496–519 and 1397–1426, respectively), produces a 303 bp product with OC cDNA from osteoblasts. OC1SP6FW and OC1T7RV primers were used for developing riboprobes for ISH. Primers for β-actin were also used for PCR internal control. The sequences of the primers used are as follows: OC1FW, cactcctcgccctattggcc; OC1RV, gccaactcgtcacagtccgg; OC2FW, gaattcatgagagccctcacactcgcc; OC2RV, tctagacta-gaccgggccgtagaagcgccgataggc; OC1SP6FW, atttaggtgacac-tata cactcctcgccctattggcc; OC1T7RV, taatacgactcactataggg gccaactcgtcacagtccgg.

2.3. Northern blotting

Human MTN Blots (Clontech Laboratories, Inc., Palo Alto, CA) were used to analyze the expression of OC mRNA in normal human tissues. A 238 bp OC cDNA was amplified with OC1 primers, cloned, and labeled with [α-^{32}P]dCTP (Amersham Biotech, Inc., Piscataway, NJ) using the random primer method (Feinberg and Vogelstein, 1983). After removal of unincorporated nucleotides, the specific activity of labeled probes was determined using a scintillation counter. Northern blotting was performed according to the manufacturer's protocol. Briefly, filters were prehybridized at 68°C for 30 min and hybridized to 1×10^6 cpm of DNA probes per milliliter at 65°C for 1 h in ExpressHyb hybridization solution (Clontech Laboratories, Inc., Palo Alto, CA). Membranes were then washed in $2 \times$ SSC/0.05% SDS at room temperature followed by $0.1 \times$ SSC/0.1% SDS at 50°C for 40 min and exposed to X-ray film at -80°C with two intensifying screens. Since the RNA quality of Clontech's MTN blots was demonstrated to be very consistent, we didn't probe the membrane with an internal control for the RNA control purpose (Brown et al., 1999; Ebrahimi et al., 1998).

2.4. RT-PCR

Total cellular RNA from each cell line was extracted by the guanidium isothiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) using an Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, TX). Total RNAs from normal human prostate, ovary, and bone marrow were purchased from Clontech Laboratories, Inc. Extracted RNA samples were treated with RQ1 RNase-free DNase (Promega Corp., Madison, WI) and with phenol-ethanol precipitation to be free of the possible genomic DNA contamination. Human Multiple Tissue cDNA Panels (Clontech Laboratories, Inc., Palo Alto, CA) were also used for identifying the isoforms of OC RNA in normal tissues.

One microgram of total RNA with 100 pM $d(T)_{20}$ was preheated at 72°C for 5 min and reverse-transcribed with M-MLV RT (Life Technologies, Grand Island, NY) at 42°C for 1 h. PCR was performed in a 50 μl solution containing 5 μl of $10 \times$ PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl), 0.2 mM dNTP mixture, 1 μl template DNA (1/20 of reverse-transcribed cDNA or 5 μl of multi-tissue cDNA), 2 mM $MgCl_2$, 0.2 μM of each OC2 primer, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). Cycling parameters were an initial denaturation at 94°C for 2 min followed by 35 cycles with each cycle at 94°C for 30 s, 63°C for 30 s, and 72°C for 2 min and the last extension at 72°C for 7 min. PCR products (5 μl) were separated by 1% agarose gel electrophoresis.

2.5. DNA sequence analysis

All DNA sequence analysis was performed with a Perkin Elmer Automatic Sequencer (Norwalk, CT) in the Core Facility Service at the University of Virginia Health Sciences Center using the dideoxy chain termination method (Sanger et al., 1977). Initial DNA characterization and translation was performed using the GCG Wisconsin version 8.0 software package (Genetics Computer Group, Madison, WI).

2.6. ISH

A segment of human OC cDNA (275 bp) was amplified using a set of promoter sequence-linked primers in RT-PCR. The SP6 promoter was incorporated into the OC1FW primer and the T7 promoter was incorporated into the OC1RV primer (Komminoth, 1992). Labeled riboprobes were synthesized by in vitro transcription of PCR-amplified cDNA using a digoxigenin-RNA labeling kit, SP6-T7 (Roche Molecular Biochemicals, Indianapolis, IN).

For the detection of OC mRNA, ISH was performed on formalin-fixed paraffin-embedded human prostate tumor tissues as described (Denijn et al., 1990; Kain et al., 1991). ISH was performed using 30 ng of probe for each slide including antisense probes, sense probes as negative controls, and β-actin probes as positive controls as previously described (Breitschopf et al., 1992). Briefly, slides were deparaffinized in Hemo-De (Fisher Scientific, Pittsburgh, PA) and washed in DEPC-treated PBS, followed by DEPC-PBS containing 100 mM glycine. After the sections were treated with proteinase K (50 μg/ml) (Roche Molecular Biochemicals) at 37°C for 30 min, 0.25% acetic anhydride (Sigma, St. Louis, MO) in 100 mM triethanolamine (v/v) was applied for 10 min. The slides were prehybridized with $5 \times$ SSC and 40% deionized formamide at 42°C for 30 min followed by hybridizing with dig-labeled riboprobes in hybridization buffer (40% deionized formamide, 10% dextran sulfate, $1 \times$ Denhardt's solution, $4 \times$ SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA) at 42°C overnight. Slides were washed two times at 50°C in $2 \times$ SSC/0.1% SDS, then in $1 \times$ SSC/0.1% SDS, and $0.1 \times$ SSC for 30 min at room temperature. Signals were then developed using the digoxigenin detection method (Herrington and McGee, 1992). The tissue distribution of OC protein was determined by immunohistochemical staining using OC 4-30 bovine monoclonal antibodies (Takara Shuzo Co., Ltd., Shiga, Japan).

3. Results

3.1. Presence of multiple isoforms of OC mRNA in normal human tissues

As shown in Fig. 1, ^{32}P-labeled OC probes were hybridized with the expected size of OC mRNA (around 450 bp) from bone marrow and, interestingly, another RNA, which is in the range of 1.0–1.2 kb in many other tissues of Human Multi-Tissue Blot. As we expected, bone marrow strongly and exclusively expressed the lower band of RNA. Trachea, small intestine, colon, and spinal cord weakly expressed both RNAs. However, most of the OC-hybridized signal was present as an upper band in the rest of the tissues, such as liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, lymph node, skeletal muscle, and peripheral

white blood cells. Some of these tissues expressed a significant amount of OC RNA. Placenta, lung, and stomach showed very low or no expression of OC.

Since the OC mRNA signal was very weak in some normal human tissues in Northern blot, RT-PCR analysis was used to verify the expression of OC transcripts. RT-PCR using OC-specific primers (both OC1 and OC2 primers) supports the Northern blot data, demonstrating that there are six different isoforms of OC RNA (Fig. 2). Most dominant signals were either 1 kb or 300 bp (expected size of OC mRNA). The smaller form of OC RNA was most dominant in small intestine, colon, and bone marrow. The reason why the smaller form of OC RNA became dominant in small intestine and colon in RT-PCR analysis likely resulted from preferential amplification of smaller fragments by PCR. In the tissues of liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, and peripheral white blood cells, the most dominant form was around 1 kb. However, lung and skeletal muscle expressed very low levels of OC, making it hard to tell which form of OC RNA was dominant. Lymph node, fetal liver, and tonsil had no expression of OC RNA. All six OC transcripts were hybridized to dig-labeled OC2 DNA probes in PCR-Southern blots (data not shown). Intermediate forms of OC transcripts (bands 2–5) may not have been detectable in less sensitive Northern blot assay and were not detected in less than 30
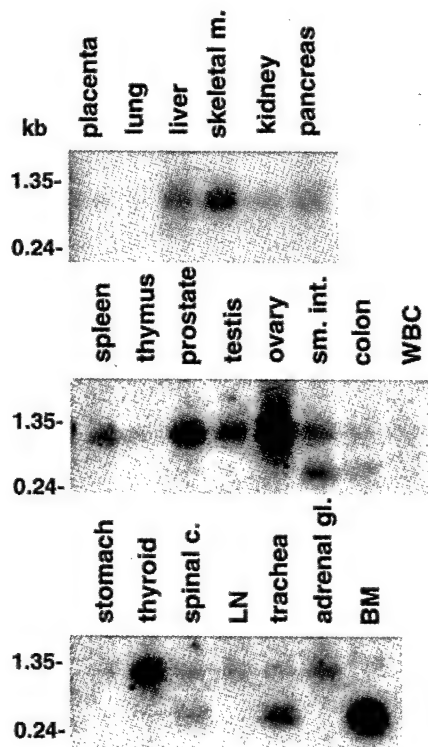

Fig. 1. Northern blot analysis of OC mRNA on human multi-tissue blot. Human MTN Blots (Clontech Laboratories, Inc., Palo Alto, CA) were hybridized with ^{32}P-labeled OC cDNA probes (238 bp), which were developed from the open reading frame of OC. After vigorous washing, membranes were exposed to X-ray film.
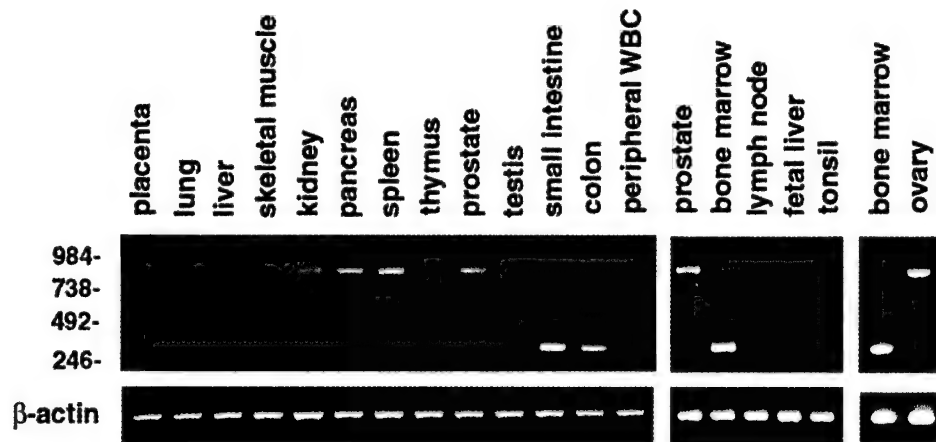
Fig. 2. Identification of multiple OC mRNA in normal human tissues by RT-PCR. Human multiple tissue cDNA (Clontech Laboratories, Inc., Palo Alto, CA) was analyzed for the presence of multiple OC mRNA by PCR (35 cycles). PCR products were run on 1% agarose gels containing ethidium bromide. Primers for β-actin were used for internal controls.

cycles of RT-PCR analysis. To exclude the possibility that the 1 kb PCR product resulted from genomic DNA contamination in cDNA, total RNA from normal prostate and heart was reverse-transcribed with or without M-MLV reverse transcriptase (Life Technologies, Grand Island, NY). No PCR products were detected without reverse transcriptase, which indicated that the 1 kb PCR product was amplified from RNA (Fig. 3).

3.2. Multiple transcripts of OC mRNA

Six different PCR products of OC from normal prostate cDNA were excised and cloned into TOPO T/A vectors (Invitrogen, Carlsbad, CA) for sequencing analysis. Fig. 4 describes the DNA structure of each PCR product of OC. Surprisingly, all five additional bands retain one or more introns and have intron 1 in common except for band 4, and the dominant one had all three introns. None of the six different RNA transcripts had any point mutations and showed 100% homology to a known human OC genomic DNA sequence except for a different combination of intron

retention. Since intron 1 is the longest intron in the OC gene, we searched for the possible amino acid codons within intron 1 and we found that intron 1 has an open reading frame with a termination codon encountered in the middle. If translated, these variant mRNAs would encode a polypeptide having 57 amino acids encoded by the 107 bases of intron 1 along with the 64 nucleotides of exon 1. This polypeptide would be a completely different form of OC variant compared to the mature OC protein, which results from cleavage at exon 3. Then, we ran protein databases to search for homologous molecules. However, no significant homology has been found in databases such as GenBank, EMBL, DDBL, and PDB sequences. Minor band 4 may encode 100 amino acids derived from exons 1–3 and intron 2.

3.3. Tissue localization of OC mRNA and protein

Since the RNAs for Northern blot and RT-PCR analyses were extracted from tissues containing multiple cell types, we conducted RNA ISH using prostate as an example to locate the specific cell types expressing OC mRNA. Due to on-hand availability, the prostate specimens used were from eight prostate cancer patients. In addition, IHC was performed to verify the tissue localization of OC protein as compared to OC mRNA. By ISH, OC riboprobes were hybridized to its counterparts both in epithelial cells (arrows) and in stromal cells (arrowheads) of prostate tumors (Fig. 5B). OC RNA expression was especially high in most of the tumor epithelial cells, whereas the OC level was very low in stromal cells. OC RNA was nonreactive to the OC sense probe (Fig. 5C). Developed from most of the OC open reading frames, OC riboprobes can be hybridized to all six transcripts identified in RT-PCR analysis. However, OC antibodies, which were developed from the N-terminal portion of mature bovine OC (γ-carboxylated residues of 15–31), were strongly reactive to stromal cells (arrowheads) and weakly reactive to a few tubular
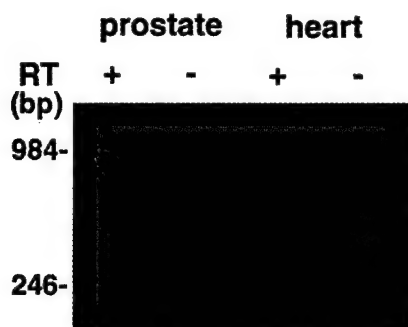

Fig. 3. OC RT-PCR products from human prostate and heart. Total RNA was reverse-transcribed with oligo (dT)$_{20}$ primer with the presence (+) or absence (−) of reverse transcriptase. cDNAs were PCR-amplified using OC2 primers and PCR products were separated on 1% agarose gel and visualized by ethidium bromide.
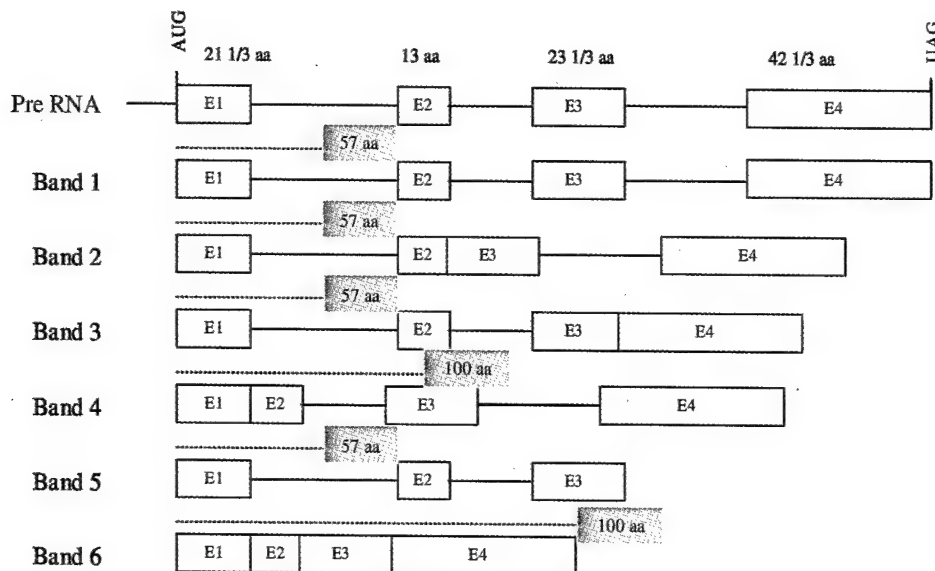
Fig. 4. A schematic organization of OC splicing variants. Exons (E) are indicated as boxes; amino acid residue numbers are indicated above. The size of predicted polypeptide variants is shown in shaded boxes.

epithelial cells (arrows) (Fig. 5D). Although bovine OC 4-30 antibodies are known to be equally reactive to both bovine and human OC (Koyama et al., 1991), we conducted both ISH and IHC on normal human bone tissues to verify the reactivity of OC 4-30 monoclonal antibodies to human OC. Both OC RNA (Fig. 5E) and OC protein (Fig. 5F) were highly reactive and restricted to osteoblasts.

3.4. Pattern of OC splicing in tumor cells

To see the difference in the level of OC RNA between the mature form and the incompletely spliced forms in the prostate cancer cell progression model, we extracted RNA from different cultured cells of prostate tumors and osteosarcomas. In MG63, a human osteoblastic osteosarcoma, mature RNA was exclusively dominant, just like normal bone marrow cells (Fig. 6). In prostate cancer cells (LNCaP, C4-2, PC3, and DU145), intron-retained OC RNAs were dominant, regardless of any prostate cancer cell progression model.

4. Discussion

We have demonstrated that OC transcriptional control might be regulated not only at the promoter level, which has been widely studied in OC research, but also by tissue-specific RNA splicing. Both Northern blot and RT-PCR assays revealed that OC intron retention occurs in non-osseous tissues. However, only bone tissues efficiently spliced OC RNA. Intron retention is a very rare phenomenon in vertebrates, although several studies have identified premature mRNA. IL-1β mRNA expression is regulated at pre-mRNA processing by keeping short-lived intron-retained transcripts (Jarrous and Kaempfer, 1994). Retention of specific intron (intron D) in growth hormone is well known in both bovine and human tissues (Hampson and Rottman, 1987; Cooke et al., 1988). In addition, CD44 gene transcripts retain intron 9. This abnormally spliced mRNA is expressed in 80% of colon carcinomas, thus implying that this variant could be linked to both tumor progression and metastatic potential (Yoshida et al., 1995; Goodison et al., 1998). Most of these studies showed tissue-specific retention of one particular intron. In the case of OC, non-osseous tissues preferentially retained all three introns.

OC is known to be exclusively expressed in bone-related tissues. Lian et al. (1989) reported that no expression of OC mRNA was detected in non-osseous tissues by Northern blot. However, RT-PCR analysis demonstrated that OC is expressed at low levels in tissues of duodenum, brain, lung, kidney, and liver (Gundberg et al., 1984). Thiede et al. (1994) also reported that platelets and megakaryocytes in rat express a moderate level of OC mRNA by Northern blot. However, human platelets express a very low level of OC mRNA and no proteins were detected in either human or rat. Here, we observed that OC is expressed in most normal tissues, although its expression level is low and transcripts are mostly expressed as an incompletely spliced form, especially in liver, kidney, pancreas, spleen, thymus, prostate, testis, ovary, and peripheral white blood cells. Combined assays of ISH and IHC support the evidence that there is a much higher expression of OC mRNA, whose probes are developed from the coding strand of mature OC, than there is of OC protein, especially in tubular epithelial cells of the human prostate. Most RNA hybridizing signals are expected to come from all six RNA transcripts. However, protein signals result from mostly mature OC (completely spliced form).
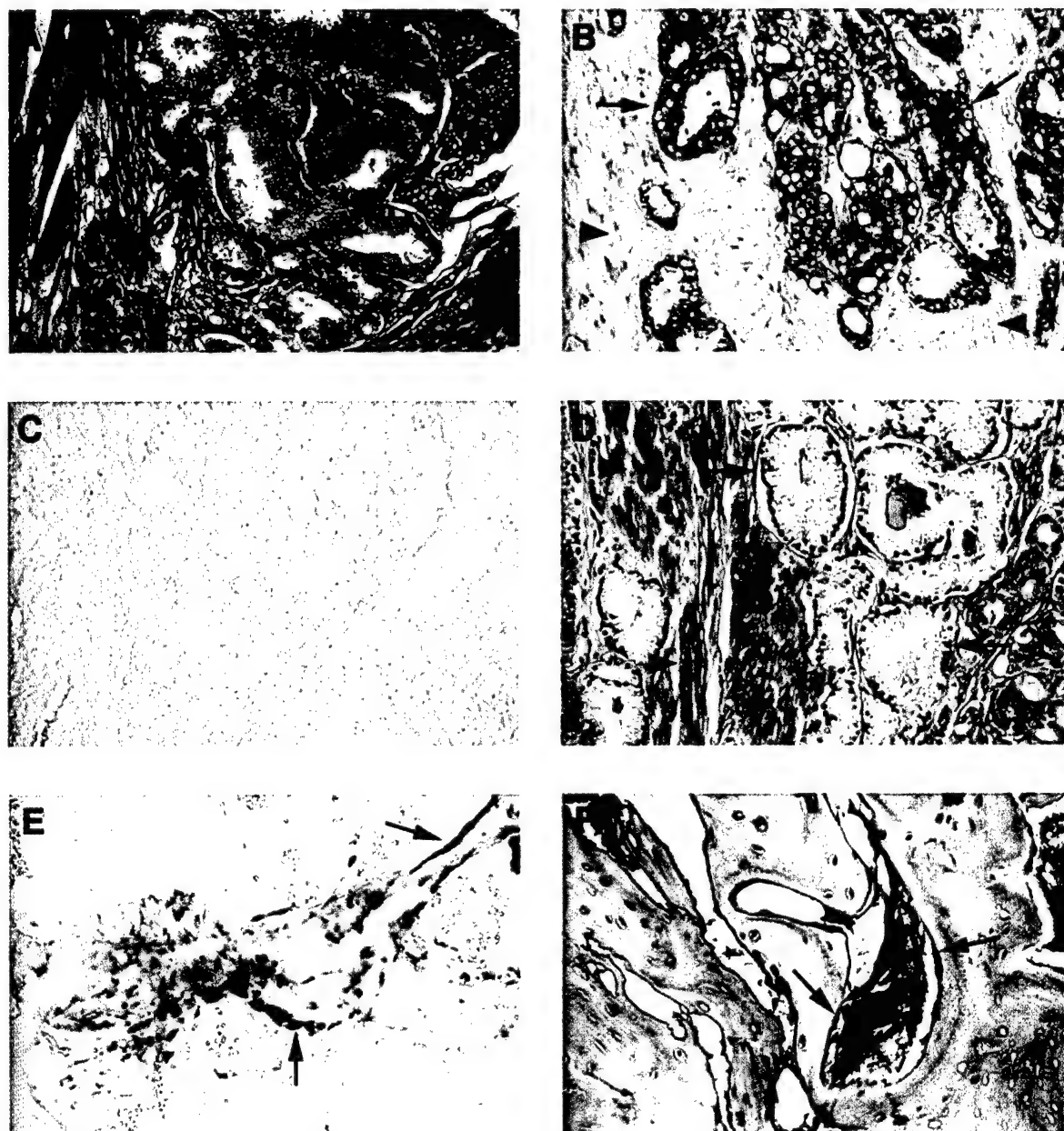
Fig. 5. Tissue localization of OC in a formalin-fixed paraffin-embedded surgical specimen of primary prostate tumor. H&E stain (A); ISH with antisense OC RNA probes (B); ISH with sense OC RNA probes (C). For IHC, tissue sections were immunolabeled with a mouse monoclonal anti-OC antibody (OC 4-30) using horseradish peroxidase (D). Normal human bone tissues were used as a positive control of OC showing either OC RNA (E) or OC protein (F). A ×10 objective was used to photograph (A–D,F), whereas a ×20 objective was used to photograph (E).

The mechanism and physiological consequence of intron retention remains unclear. Most studies suggest that intron retention in mRNA depends on a balance of the strengths of suboptimal splice sites and of the presence of an exonic splicing enhancer in the adjoining exon (Dirksen et al., 1995; Robberson et al., 1990). However, intron retention of OC mRNA in non-osseous tissues possibly employs a different mechanism. All pre-mRNA $5'$- and $3'$-splice sites of the three introns conformed to the consensus dinucleotide sequences gt and ag, respectively, and all introns have a consensus sequence of the branching site.

The mouse OC gene cluster contains three structural genes arranged within 23 kbp of genomic DNA in the same transcriptional orientation (Desbois et al., 1994; Rahman et al., 1993). These genes are osteocalcin gene 1 (OG1), OG2, and osteocalcin-related gene (ORG). OG1 and OG2 have 98% homology in coding sequence and have a six-nucleotide substitution in exon 1. ORG and OG1/OG2 have 96% homology but an additional non-coding exon at the $5'$-end. OG1 and OG2 genes are specifically expressed in bone while the ORG gene is the only form of OC expressed in other tissues, but not in bone (Rahman et al.,
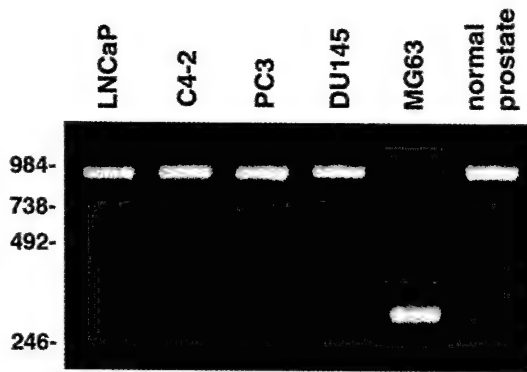
Fig. 6. Demonstration of OC products in selected cell lines by RT-PCR. Total RNA was isolated and reverse-transcribed as described. Resulting cDNA was amplified using OC2 primers followed by agarose gel analysis.

1993). However, the genomic DNA structure of human OC has not been revealed and it should be further investigated as to whether OC RNAs detected in different tissues result from different genes. Duplication of the PSMA gene under a different tissue-specific promoter has also been reported (O'Keefe et al., 1998). Nevertheless, ORG involvement in human tissues might not be the explanation of the tissue-specific splicing of OC RNA since the OC RNA sequence from normal prostate is 100% homologous to that of bone.

Both RT-PCR and Northern blot analysis revealed that only bone-related tissues acquire the phenomenon of complete splicing. In addition, combined assays of ISH and IHC in primary prostate tumors demonstrated that completely spliced OC becomes the dominant form when tumors metastasize to the bone (data not shown). These results lead us to predict that bone tissues may provide the proper environment to complete OC splicing and there may be a bone tissue-specific splicing factor(s). The presence of tissue-specific splicing factors has been recently identified. An alternative splicing phenomenon in neuronal cells is dependent on a neural-specific 75 kDa protein, which interacts with the intron regulatory element (Min et al., 1995). In addition, GnRH is incompletely spliced in non-neuronal cells and neuron-specific splicing factors are proposed (Seong et al., 1999). Nevertheless, further research has to be performed to explore the hypothesis that bone-related tissues have bone tissue-specific splicing factor(s).

The functional significance of the intron retention of OC in various normal tissues is another area to be clarified. OC is known to be a negative regulator of tissue mineralization and bone turnover so that OC protein is not expressed during the bone development of fetus (Ducy et al., 1996; Luo et al., 1997). Therefore, the results of this study suggest that low levels of completely spliced OC and its resultant protein may keep normal tissues from pathological calcification. However, the majority of incompletely spliced OC can be converted to the mature form in certain circumstances, such as in the bone environment, and this leads the corresponding tissue to pathological status. On the other hand, since

incompletely spliced transcripts consist of exon 1 and intron 1, coding for a novel OC protein with 57 amino acids if translated, it might be interesting to investigate whether this protein has a biological function or not. It is also interesting to see whether this splicing control is involved in diseased tissues showing calcification.

In conclusion, this study identified OC mRNA isoforms, which are exclusively present in non-osseous tissues, suggesting the presence of bone tissue-specific splicing factor(s). Further work is in progress towards the in vitro characterization of OC splicing and identification of the splicing factor(s) in bone-related tissues. This will allow us to explain the mechanism of differential splicing of OC and to demonstrate transcriptional regulators on OC expression, rather than promoter-based regulation.

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Osteocalcin-directed gene therapy for prostate-cancer bone metastasis

Abstract Osteocalcin (OC) is a major noncollagenous bone protein whose expression is limited almost exclusively to osteotropic tumors and mature calcified tissue (differentiated osteoblasts). The function of OC, a highly conserved gamma-carboxyglutamic acid-containing protein, relies in part on its ability to bind hydroxyapatite and act as a chemoattractant for bone-resorbing cells. Serum osteocalcin levels are used clinically as an index of active bone turnover. Research in our laboratory has revealed that OC is expressed in several solid tumors, including osteosarcoma and ovarian, lung, brain, and prostate cancers. Evidence arising from reverse-transcription polymerase chain reaction (RT-PCR; detection of OC mRNA), immunohistochemical staining (detection of OC protein), and transient transfection and reporter assay (detection of OC mRNA transcription) reveals that OC expression is up-regulated in numerous solid tumors, with its expression being further elevated in androgen-independent prostate cancers. A recombinant, replication-defective adenovirus, Ad-OC-TK (OC promoter-driven herpes-simplex-virus thymidine kinase) was constructed and, when combined with the appropriate prodrug, either ganciclovir (GCV) or acyclovir (ACV), was found to be effective at destroying prostate-cancer cell lines in vitro and prostate tumor xenografts in vivo in both subcutaneous and bone sites. Additionally, via use of the OC promoter the supporting bone stromal cells are cotargeted when the prostate cancer interdigitates with bone stroma at the metastatic skeletal sites. Thus, maximal tissue-specific cell toxicity is achieved by the interruption of cellular communication between the prostate cancer and the bone stroma. We describe herein the preclinical foundation as well as the design and implementation of an ongoing phase I clinical trial at the University of Virginia that targets androgen-independent metastatic prostate cancer using the Ad-OC-TK vector.

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Over the past several years, our laboratory has focused attention on elucidation of the molecular mechanisms underlying stromal-epithelial interaction in prostate carcinogenesis [6, 7]. In a series of studies, it was demonstrated that a critical interaction between prostatic epithelium and organ-specific stromal cells accounted for the androgen-independent progression of prostate cancer and the acquisition of metastatic potential to the lymph nodes and skeleton [17, 35]. Studies in our laboratory as well as others have clearly documented that either soluble growth factors or extracellular matrices play key roles in affecting cancer epithelial growth and differentiation. Interruption of this reciprocal cellular interaction between prostate-cancer epithelium and stroma abrogates cancer-cell growth and delays androgen-independent disease progression [33]. This plan of attack allows the targeting of stroma matrix elements coexpressed by prostate cancer and bone stroma, such as osteocalcin (OC). The impetus behind this work has been carried by Leland W.K. Chung and Haiyen E. Zhau over many years. Much of the substantial preclinical investigation was done by Song-Chu Ko and Chinghai Kao as well as many other investigators [16]. Urologists Thomas A. Gardner and Jay Y. Gillenwater spea- ·

headed the drive to make the described phase I trial of adenovirus Ad-OC-TK [OC promoter-driven herpes-simplex-virus (HSV) thymidine kinase] a reality, with substantial input being provided by David A. Kallmes of the Department of Radiology. In this report we propose to test four hypotheses that could lead to significant advances in our understanding of the responsiveness of prostate cancer to toxic gene therapy.

Hypothesis I: stromal–epithelial interaction is a key determinant of prostate-cancer growth and progression

Interruption of the stromal-epithelial interaction using a common promoter shared by these two cellular compartments to drive the expression of a toxic gene, HSV-TK, in the presence of a prodrug [an orally active acyclovir (ACV) analog, e.g., valacyclovir] could result in efficient cell kill in vivo.

Hypothesis II: antitumor immune response is essential for Ad-OC-TK/Val-induced cytotoxicity in tumor epithelium and its supporting stroma

Immunologic response to cytotoxic gene therapy in prostate cancer is a nascent field without substantial prior investigation, especially in humans. Numerous investigators have implied a role for the immune system, with both local and systemic effects resulting from TK-expressing gene therapy [18], although the candidate effector cell(s) mediating these activities remain(s) unproved. Evidence indicates that tissue macrophages and natural killer(NK) cells are intimately involved in the immune response to TK-mediated gene therapy [19, 30]. Indeed, evidence is accumulating that TK-gene-mediated killing can be augmented by sustained antitumor immunity, whether via endogenous or facilitated cytokine expression [4, 34]. Apoptosis in prostate cells has been associated with activated macrophages and cytotoxic T-cells [25]. By studying the nature of the immunologic response generated in this phase I trial we hope to identify prognostic factors present in either responders or nonresponders and to incorporate immunologic elements with suicidal toxic genes cotargeting tumors in future vector designs.

Hypothesis III: biomarkers and novel imaging application/techniques may predict Ad-OC-TK/ACV-induced cytotoxicity and clinical response in patients treated with Ad-OC-TK/Val gene therapy

Tumor response to therapy can be broadly defined as the inhibition of cell proliferation and/or enhancement of tumor apoptosis. The overall apoptotic indices can be reflected by the degree of DNA fragmentation and strand breakage and factors involved in activation of apoptotic cascades (bcl-2, bax, bcl-xl, caspase-3). These factors can be immunohistochemically evaluated and quantitatively assessed by reverse-transcription polymerase chain reaction (RT-PCR).

Lastly, we propose to integrate both traditional and emerging morphologic imaging techniques [radiography, computed tomography (CT), and magnetic resonance imaging (MRI)] and function studies [bone scintigraphy and (FDG-PET)] to characterize the response of patients with osseous metastasis treated by cytotoxic gene therapy. Evaluation of these biomarkers and imaging techniques would improve our understanding of the array of response parameters associated with the best predictive value for this new therapeutic modality. For example, the responding patients may all have limited axial skeletal metastasis, very high tissue indices of apoptosis status postinjection, and significant changes pre- and posttherapy on MRI and FDG-PET scans, indicating a synergistic effect of the suicide-gene therapy upon both the epithelial and stromal cellular compartments as augmented by host antitumoral immunity. Any biomarker that appears to predict the response to therapy will be used in subsequent trials (phase II/III) to direct the most efficacious patient accrual and monitoring.

Hypothesis IV: Ad-OC-TK/Val therapy is most efficacious in inducing tumor regression at the skeletal metastatic site

OC, a noncollagenous Gla protein associated with prevalent skeletal-tissue and selected solid-tumor expression, should allow the Ad-OC-TK strategy to yield the most promising results in prostate-cancer skeletal metastasis.

Osteomimetic properties of prostate cancer

Bones form by endochondral or membranous ossification during embryonic development and are subsequently remodeled during adult life. Bone remodeling involves an interaction between stromal and hematopoietic cells to ensure the removal of trenches of worn-out bone from bone surfaces by osteoclasts and their subsequent replacement by new bone laid down by osteoblasts [29]. Bone composition is a homeostasis between osteoblastic production and osteoclastic remodeling. These highly regulated processes determine the size, shape, and strength of bones and are under the control of locally released factors that direct the proliferation, activity, and life span of bone cells. Recent studies in knockout and transgenic mice have identified a number of factors required for bone-cell formation, activity, and survival [3, 9, 22]. Thus, a balance exists between proliferation and apoptosis.

Like the prostate, bone is quite sensitive to hormone and growth factors. Some of the key elements in bone

homeostasis are the noncollagenous proteins. At least 12 different noncollagenous matrix proteins have been identified as major constituents of bone and teeth. These noncollagenous proteins are made and secreted by pre-osteoblast and osteoblast cells in a distinct spatially and temporally defined manner [37]. Osteoblasts produce the collagenous matrix, which mineralizes to form woven or lamellar bone. Uniquely, prostate-cancer osseous metastases, like osteosarcoma, have been described as "osteosclerotic" and "osteoblastic" [36]. Other cancers produce osteolytic lesions.

The molecular composition of this rapidly deposited and mineralized bone associated with prostate-cancer metastasis most closely approximates "woven" bone. Woven bone is primary bone formed de novo by the process of intramembranous ossification, by which bone is formed directly by cells of mesenchymal origin without cartilaginous precursors. Woven bone can form in 2–3 weeks, whereas lamellar bone may take 3 months [15, 26]. Woven bone displays a lower mineral density, higher water content, and more disorganized packing of collagen than does lamellar bone. Uniquely, woven bone can be completely removed by osteoclasts, whereas lamellar bone cannot. This is evident in prostate-cancer bone metastases, which disappear upon androgen ablation. Prostate carcinoma most commonly metastasizes to the lymph node and bone, showing a 70–80% frequency in autopsy studies [14, 27]. Metastasis to the bone by prostate adenocarcinoma most commonly involves the vertebrae, followed in order of frequency by the sternum, pelvic bones, ribs, and femurs [20]. This proclivity for bone metastasis has been explained by Batson's "plexus retrograde flow" theory [1, 2] and by Paget's "seed and soil" theory [28].

Recently we have expanded the mechanistic understanding of prostate-cancer bone tropism and metastasis by outlining how prostate-cancer cells become "osteomimetic" [24]. This "osteomimetic theory" demonstrates *common utilization* by bone stroma and prostate epithelium of growth factors, adhesion molecules, matrix proteins, transcription factors, and circulatory soluble factors (osteotropic hormones). Below we describe the ongoing phase I clinical trial as an initial attempt to treat hormone-refractory, androgen-independent metastasis of prostate cancer on the basis of preclinical investigations using a molecular understanding of the osteomimetic properties of prostate-cancer cells.

Preclinical background

The mouse osteocalcin (OC) promoter is tissue-specific and drives the expression of OC protein primarily in osteoblasts [10, 11, 21]. OC expression is associated with bone formation, calcification, and microcalcification associated with cancer-cell transformation. Endogenous OC mRNA can be assessed by RT-PCR, whereas protein expression can be detected by immunohistochemistry, Western blotting, and enzyme-linked immunosorbent assay (ELISA). RT-PCR reveals OC product in rat liver, kidney, duodenum, lung, bone, and brain, with the level of OC mRNA determined in bone being 1000-fold that detected in other tissues [13]. OC promoter-directed transgene expression (OC-CAT) in mice demonstrated activity in the calvaria, femur, cartilage, and bone marrow and a small amount in the brain. Immunohistochemistry (IHC) in primary and metastatic prostate cancer (lymph node and bone) has demonstrated homogeneous staining. RT-PCR has demonstrated OC in human prostate cell lines and in prostate tissues. Mouse OC-promoter activity as measured by reporter-gene or therapeutic gene expression has been well documented in human prostate-cancer cell lines in our laboratory.

Adenovirus vectors used in the present study belong to subgenus c, serotype 5 (Ad5). Wild-type Ad5 results in acute mucous membrane infection of the upper respiratory tract, eyes, lymph glands, and nose associated with symptoms similar to those of the common cold. Exposure to c-type adenoviruses is widespread, and a majority of adults are serum-positive for this virus. Adenoviral protein expression occurs in two major phases, early and late. In general, early proteins are regulatory in nature, whereas late proteins are structural. Replication-defective adenoviruses capable of transducing cancer cells in vitro and in vivo with desired therapeutic genes have allowed the initiation of several human adenoviral gene-therapy trials [8, 12, 31].

The target gene involved in the preclinical work and trial is thymidine kinase from the herpes simplex virus (HSV-TK). HSV-TK has greater affinity for the purine analogs ganciclovir (GCV) and acyclovir (ACV) than does the mammalian form of TK. The affinity of these analogs for viral TK explains the clinical efficacy of these antiviral therapeutics. Cancer cells transfected with HSV-TK can be killed by the administration of a prodrug (purine analog). HSV-TK codes for an enzyme that phosphorylates a guanine analog, either GCV or ACV. The phosphorylated product can be incorporated into newly synthesized DNA, leading to DNA chain termination and apoptotic cell death. "Bystander" experiments demonstrate that < 100% cell expression leads to significant cell kill. This TK prodrug paradigm has previously been used with universal promoters [cytomegalovirus (CMV), Rous sarcoma virus (RSV)] and shows early promise. Following direct tumor injection (intralesional) the majority of cells, dividing or nondividing, that are infected by the virus and possess the potential to express HSV-TK will be tumor cells. When the mouse OC promoter is placed in front of the HSV-TK gene, the TK enzyme will be expressed only in cells capable of activating the OC promoter (osteoblasts and prostate epithelium). Intralesional injection exposes adjacent normal cells to the virus, and some systemic delivery may occur. The normal cells must be capable of activating the OC promoter and must also be undergoing cell division to be adversely affected by the HSV-TK gene.

Valacyclovir, an L-valine ester of ACV, provides significantly greater oral bioavailability and convenient twice-daily (b.i.d.) dosing. Valacyclovir is converted to ACV by intestinal and hepatic enzymes. Both ACV and valacyclovir are safe and efficacious prodrugs, equivalent to GCV in our hands. GCV has been used for cancer gene therapy with the HSV-TK gene but requires intravenous administration b.i.d. due to minimal oral bioavailability.

The Ad-OC-TK construct contains a portion of the mouse OC promoter linked to the HSV-TK target gene in the E1-deleted region of Ad5. Ad-OC-TK was constructed via homologous recombination in transformed human embryonic kidney 293 cells by a common method (see Fig. 1) using a shuttle vector and recombination vector. This vector, Ad-OC-TK, can drive therapeutic toxic gene (TK) expression in prostate tumors, normal osteoblasts, and osteosarcomas, whereas some non-prostate-cancer cells and normal cells do not express TK activity. Using this tissue-specific and tumor-restricted promoter to drive a suicide gene that requires cell division for death, this gene-therapy approach doubly protects normal mammalian cells. Cell death should occur in proliferating tumor and osteoblastic cell compartments without affecting nondividing normal osteoblasts.

Preclinical toxicity tests in C57/BL mice demonstrate minimal ill effects, if any, from direct intraosseous injection of Ad-OC-TK into the distal femur. Since the vector is injected into the marrow space, some systemic delivery occurs. Conversely, as expected, universal promoter-TK constructs produce severe hepatotoxicity in test animals in our studies; in addition, liver toxicity has been confirmed by other investigators using universal promoters with HSV-TK adenovirus vectors. Use of the Ad5 virus with the HSV-TK target gene has been approved by the NIH Recombinant DNA Advisory Committee and the Food and Drug Administration in multiple clinical trials. The OC promoter has not yet been used in human trials, but several publications from our laboratory demonstrate a marked inhibition of the growth of osteosarcoma by either intralesional or systemic administration of Ad-OC-TK followed by the administration of ACV [5, 23, 32]. The approach outlined in this report, using the intralesional Ad-OC-TK vector, should improve the safety of suicide-gene therapy by limiting gene transcription to only the targeted prostate-cancer epithelium and/or the supporting active stroma of osteoblastic lineage.

Trial design

The primary objective of this phase I study was to enroll 12–18 patients for evaluation of the safety of this novel gene therapy. A secondary objective was to confirm the biologic feasibility and evaluate the potential efficacy of this therapy for the treatment of patients with metastatic or locally recurrent prostate cancer. Replication-defective human adenovirus, Ad-OC-TK, is used to treat men with hormone-resistant metastatic or locally recurrent prostate cancer. The metastatic lesion must be radiologically measurable and amenable to computed tomography (CT)- or ultrasound-guided injection. Only one index lesion per patient is injected. An investigative team of urologists, oncologists, and radiologists evaluates patients meeting the entrance criteria. The protocol specifies the administration of an escalating viral dose beginning at 2.5×10^8 plaque-forming units (pfu)/injection in a volume of 0.5–2 ml, depending on the tumor volume, at the index lesion. Each patient receives two injections, one on day 1 and one on day 8. After each injection of virus the patient is hospitalized for 48 h. Valacyclovir (b.i.d.) is started at the first injection and continued for a total of 21 days. On both day 1 and day 8, prior to Ad-OC-TK injection, at least two to three 18-gauge core biopsies are taken from the index lesion. An index-lesion biopsy is again taken on day 30 of the study. Some of this material is saved "fresh" in liquid nitrogen, some is used to produce cell lines in culture, and the majority is paraffinized for immediate pathologic evaluation and future marker evaluation. Dose escalation is completed according to the following schedule: three patients are given the low dose, three patients are tested at the intermediate dose
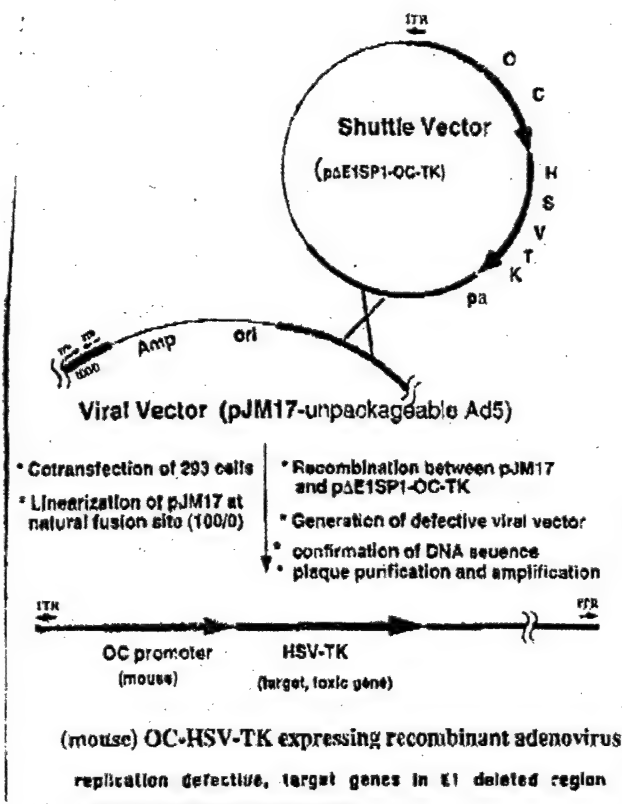

Fig. 1 Schematic representation of the construction of Ad-OC-TK via homologous recombination in embryonic kidney 293 cells. Mouse OC promoter linked to the HSV-TK target gene is inserted in the E1-deleted region of the Ad5 virus

$(2.5 \times 10^9$ pfu), and the remainder are given the high dose $(2.5 \times 10^{10}$ pfu). Each patient is carefully monitored for cytopathic and toxic effects throughout the therapy. This monitoring includes the performance of numerous types of routine blood work – such as CBC with differential, liver-function tests, determination of prothombin time/partial thromboplastin time (PT/PTT) and of blood urea nitrogen (BUN)/creatinine, serum chemistry, determination of prostate-specific antigen (PSA) and of prostatic acid phosphatase (PAP), and urinalysis – throughout the study but more frequently during the initial 30 days. Virus-shedding studies are done prior to and during the therapy along with determination of serum viral titers and serum OC levels.

All patients undergo multiple imaging modalities prior to and during the study. This includes chest X-ray, radionuclide scintigraphy (bone scan), CT of the abdomen/pelvis, MRI of bone lesions and "whole-body" bone-marrow survey, and, when indicated, transrectal ultrasound (TRUS) of the prostatic fossa. In addition, positron emission scanning (PET) is done on selected patients. The combination of anatomy/morphology and function studies allows the evaluation of treated and untreated metastatic sites along with correlation with tumor-associated parameters before and after adenoviral gene therapy. Since all patients enrolled must have bidimensionally measurable index lesions, the efficacy of the therapy can be estimated. Additional measures of efficacy include surrogate endpoints such as serum tumor markers (PSA, PAP, alkaline phosphatase) and pain relief. An extensive quality-of-life questionnaire that evaluates multiple parameters is also included in the protocol.

The entrance criteria include common parameters such as stabilization of the pain-medication dose, failure or refusal of hormone ablation, an Eastern Cooperative Oncology Group (ECOG) performance status of ≤2, the absence of a concomitant protocol drug, and the ability and willingness to give informed consent. In addition, normal liver and renal function as well as a normal platelet and coagulation axis (PT/PTT) are required along with adequate blood-count parameters (e.g., hemoglobin ≥8.5 mg/dl, absolute neutrophil count > 1000/mm³).

Progress report

The low- and intermediate-dose cohorts (3 men at each dose level) completed the injection phase without clinical complications; a total of 11 men, including 5 from the high-dose cohorts, completed the early part of the injection phase of the trial (~30 days). The age range for the study population is 53–77 years. The geographic distribution includes Tennessee, New York, Maryland, and Virginia. According to the protocol, all subjects have metastatic or locally recurrent prostate cancer. Often the patients have failed surgery, radiation, androgen blockade, and conventional chemotherapy. All have had histologic confirmation of prostate cancer at

the index lesion by biopsy. All patients display disease progression, having either failed hormone-deprivation therapy or consistently refused hormonal ablation.

The index-lesion sites that were injected with Ad-OC-TK on two occasions include two locally recurrent umors in the prostatic bed following surgery and radiation, four cases of retroperitoneal lymph-node metastasis following surgery and/or radiation, and five cases of osseous metastasis involving the L2 spine $(n = 2)$, the L3 spine $(n = 1)$, the left ilium $(n = 1)$, and the left ilium/ischium $(n = 1)$. Figure 2 displays the bone scan (anterior and posterior), sagittal spine MRI image (T1-weighted, showing hypointensity in the tumor-bearing area), and CT scan (L2 biopsy and Ad-OC-TK injection) of a patient. For purposes of comparison, Fig. 3 demonstrates a metastatic deposit in the left ilium on a bone scan, a T2-weighted MRI image of a coronal "bone-marrow survey," and biopsy/injection on a transverse CT scan. The left iliac prostate-cancer metastasis illustrated in Fig. 3 appears as a hyperintense area on this T2-weighted MRI "survey." Lastly, Fig. 4 depicts a left retroperitoneal node immediately after biopsy, just prior to Ad-OC-TK injection. Pretherapy PSA levels ranged from 0.98 to 170 ng/ml. As expected in this population, some patients were high-level PSA producers, whereas others showed very low values for the tumor burden carried. An outline of the treated patients is shown in Table 1.

As predicted from the preclinical studies and toxicology investigation, the OC promoter has displayed its true tissue- and tumor-restricted nature. The absence of any clinically relevant toxicity has been the rule. No hepatotoxicity has been seen, despite strong evidence of transient systemic circulation of the virus. Both of the patients with locally recurrent tumors have displayed virus transiently in their urine without sequelae. The most consistent observation has been a brief (≤24 h) "flu-like" syndrome involving fever, occasional chills, and fatigue along with rare episodes of myalgia and arthralgia; consistent with this finding, brief decreases in lymphocyte counts have been encountered for ≤72 h. The relative paucity of ill effects in comparison with the extensive toxicity of conventional chemotherapy is encouraging and demonstrates the bright outlook for patients when molecularly designed therapeutics are employed in the future.

Endpoint evaluation

Since the present investigation is an ongoing trial, no specific mention of efficacy is appropriate at this time. The data need to be analyzed carefully and used for the construction of a phase II trial. Key to the analysis of these data is efficient use of the precious biopsy material pre- and posttherapy; this material includes fresh frozen samples and abundant paraffin-tissue blocks. The data can then be correlated with laboratory and radiographic markers for the determination of gene transduction and
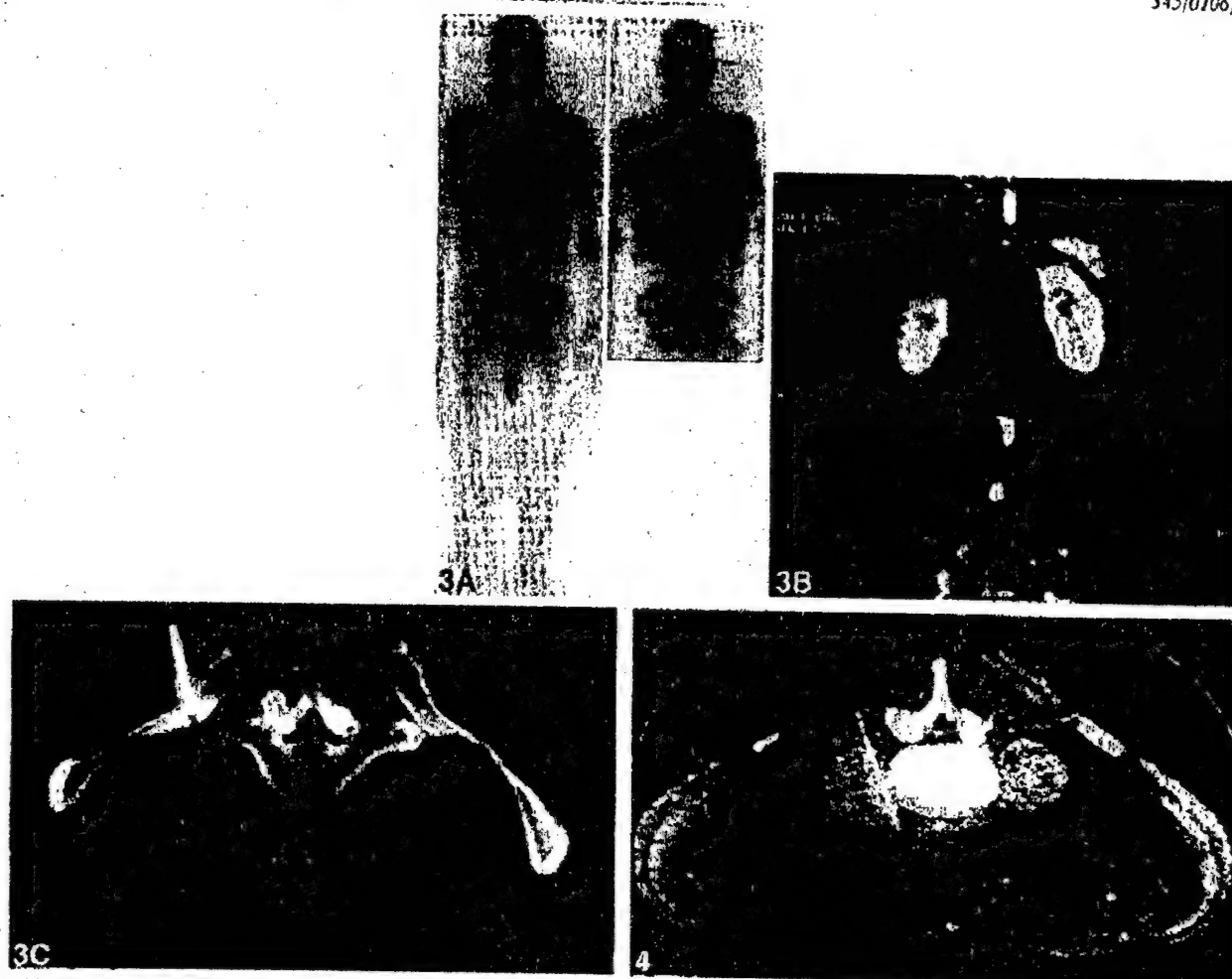
Fig. 3A–C This patient has an osteoblastic metastatic focus in the left ilium. A Pretherapy bone scan showing a hyperdense area of interest in the left ilium. The spinal hyperintense area corresponds to degenerative disease associated with an area of previous surgery. B T2-weighted MRI image of a coronal "bone-marrow survey," with the afflicted hyperintense metastasis again being visible in the left ilium. C CT-guided biopsy and Ad-OC-TK injection of this region

Fig. 4 Transverse CT scan revealing an enlarged left retroperitoned lymph node at the time of biopsy and injection of Ad-OC-TK. The needle tract transverses the psoas muscle

are growing in culture. The basal and Ad-OC-TK/Val-induced antiadenoviral antibody titers in sera from patients treated with adenoviral gene therapy are known. Ad-OC-TK may enhance antitumor immunity in the injected recurrent primary and metastatic sites. Such antitumor immunity may be time- and site-dependent. The titer of circulating antiadenoviral antibodies in sera may be inversely correlated with the response to the Ad-OC-TK/Val therapy. The WBC "buffy coats" of blood specimens have been archived from pre- and posttherapy blood specimens and will be used for future investigation.

Aim 3 is to obtain clinical correlates in tumor and blood specimens obtained from patients before, during, and after Ad-OC-TK/Val therapy. Two main analyses will be performed:

1. To determine the degree of apoptosis induced in prostate-cancer specimens obtained from primary or metastatic sites. The results will be evaluated by ■ (TUNEL) assay.
2. To conduct novel radiographic imaging evaluation of tumor sites and untreated lesions in patients subjected to Ad-OC-TK/Val toxic gene therapy.

The hypotheses to be tested in these analyses are:

1. Whether Ad-OC-TK/Val treatment causes apoptosis in both tumor epithelium and its adjacent stroma. A positive relationship between depressed serum PSA levels and enhanced apoptosis may be established.
2. Total-body MRI and ^{18}F-labeled deoxyglucose-PET scans may improve the imaging of metastatic lesions and their responses to toxic gene therapy and may be correlated with biomarkers and immunohistochemistry data obtained from biopsy specimens.

Aim 4 is to test the hypothesis that the bone-metastasis group will be the group most responsive to Ad-OC-TK/Val therapy. The overall long-range goals of this

Fig. 2A–C Three radiographic images depict the pretherapy L2 osseous metastatic region of a patient in the high-dose group. A Radionuclide scintogram. B Tl-weighted sagittal spine MRI. C Transverse CT scan obtained during biopsy and injection of Ad-OC-TK

efficacy. Four main specific aims for serum, biopsy, and radiographic imaging are under study; these address the hypotheses presented at the beginning of this report.

Aim 1 is to determine the levels and distribution of Ad-OC-TK expression in prostate-cancer cells at recurrent primary and metastatic lymph-node and bone sites. Specifically, we are evaluating the expression of Ad-OC-TK in both the tumor epithelium and the host stromal component (including but not limited to fibroblasts, osteoblasts, and pericytes). Both the distribution and the residence of adenoviral particles in recurrent primary and metastatic specimens will be evaluated by immunohistochemistry. Levels of therapeutic toxic gene (HSV-TK) expression will be evaluated in biopsy-tissue specimens by immunohistochemical assay. The adenoviral vector's distribution and ability to replicate in cells is being evaluated by PCR and bioassays in blood and urine specimens obtained from men within 1 month of Ad-OC-TK/Val administration. The hypothesis to be tested is whether Ad-OC-TK is expressed during the early period of infection (<4 weeks) in the cancer epithelium and stromal component. Depending on the site of tumor growth, specific cell types in the stromal

compartment, such as osteoblasts and pericytes (mesenchymally derived stem-cell populations with osteogenic potential), may express Ad-OC-TK activity. Surrogate biomarker activity, as a result of destruction of these mesenchymal cells and tumor epithelium, may serve as a predictive factor for cancer regression induced by toxic gene therapy. Immunohistochemical staining of the viral particle and TK enzyme may be markedly reduced in prostate cancer and its surrounding stromal component at 1 month after initial adenoviral toxic gene therapy. Immunohistochemical staining of biopsies is being done for OC and other bone noncollagenous proteins, such as osteonectin (OSN) and bone sialoprotein (BSP), and the findings are being correlated with the results obtained in hematoxylin and eosin (H&E)-, PSA-, and TK-stained sections.

Aim 2 is to assess and compare Ad-OC-TK-induced antitumor immunogenicity in tumor specimens obtained from recurrent primary, lymph-node, and bone sites. Immunohistochemical evaluation of CD-4+, CD-8+, class I major histocompatibility complex (MHC), the antigen-transporter proteins TAP-1 and TAP-2, and transforming growth factor-β1 (TGF-β1) activity associated with tumor-biopsy specimens obtained from the primary and metastatic sites will be carried out and the results will be compared. In addition, when appropriate, we will evaluate and compare the above-mentioned parameters in cell strains obtained from the tumor specimens. Currently, tumor cells from at least eight patients

Table 1 Overview of the patients enrolled in the phase I Ad-OC-TK gene-therapy trial at the University of Virginia. At the time of publication, all patients had been injected with recombinant virus ≥6 weeks previously and were without clinical complications

Patient	Dose	Index site injected	Pretherapy PSA (ng/ml)	Follow-up
1	Low (2.5×10^8 pfu)	Local prostatic fossa	9.00	≥120 days
2	Low (2.5×10^8 pfu)	Lymph node	170.00	≥120 days
3	Low (2.5×10^8 pfu)	Lymph node	19.52	≥120 days
4	Intermediate (2.5×10^9 pfu)	L3 spine	0.98	≥90 days
5	Intermediate (2.5×10^9 pfu)	Ischium/ilium (bony pelvis)	160.00	≥90 days
6	Intermediate (2.5×10^9 pfu)	Local prostatic fossa	2.70	≥90 days
7	High (2.5×10^{10} pfu)	Lymph node	83.00	≥40 days
8	High (2.5×10^{10} pfu)	Ilium (bony pelvis)	45.00	≥40 days
9	High (2.5×10^{10} pfu)	Lymph node	21.00	≥40 days
10	High (2.5×10^{10} pfu)	L2 spine	14.00	≥40 days
11	High (2.5×10^{10} pfu)	L2 spine	128.00	≥40 days

treatment are (1) to treat and/or delay the progression of prostate cancer to widely disseminated disease; (2) to improve pain relief; (3) to improve the quality of life of patients diagnosed with metastatic skeletal disease; (4) to demonstrate the lack of toxicity of injection of the adenoviral vector, designed such that its expression is tightly controlled in a tissue-specific and tumor-restricted manner, into bony metastatic lesions (which essentially represents a systemic injection); and (5) to explore and gain an understanding of all the pathophysiologic implications of Ad-OC-TK/Val toxic gene therapy. The safety and efficacy data gathered in this initial phase I trial of intratumoral injection will be used in future trials involving the systemic injection of a "retargeted" virus, which will "home" to desired neoplasia, thereby circumventing possible toxicity to vital organs.

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Appendix 3

VII. SUMMARY

The adenovirus vector, H5-OC-TK, which contained the herpesvirus thymidine kinase (TK) gene driven by the osteoblastic specific osteocalcin (OC) promoter was examined for its potential toxicity in combination with Valtrex (the prodrug acyclovir) administration in C57BL/6 mice. Vector was delivered in three doses, including 2.5×10^7, 2.5×10^8, or 2.5×10^9 plaque forming units (pfu) in 25 ul into the medulla of the right femur of C57BL/6 mice on Day 1 after surgical incision through the skin at the intended injection site. The vector instillations were repeated at Day 8 to the same groups (Two control groups received saline via intramedullary injection into the right femur, with half receiving Valtrex from Days 2 through 21 and half receiving no further treatment). Valtrex was delivered intraperitoneally from Days 2 through 21. At each of three necropsy days (5, 22 and 47), blood was taken and analyzed for blood cell evaluations (CBCs and differentials) and liver function tests. The animals were also observed daily for any clinical symptoms and gross pathology at necropsy. Finally, a number of tissues were taken for microscopic examinations of fixed and stained sections. Almost none of the study tissues for Day 22 could be evaluated histologically for toxicity as a result of the autolysis (almost no nuclear detail left in any slide). The cause of the autolysis was not certain, but improper fixation was the likely cause.

Overall the vector and Valtrex combinations were well tolerated, as there were no obvious clinical signs or body weight differences compared to the control animals. There were a number of toxicities noted that appeared to be related to the instillation process as they were present in the groups receiving vector and Valtrex, Valtrex alone, and the saline control: some deaths during the surgical instillation and within a 24 hour period following; some gross pathological changes at the site of injection; and histological changes at the femur site of injection. The histological changes ranged from fibrosis at early timepoints to more chronic lesions at the Day 47 necropsy. Most of the blood cell counts and liver function tests were not affected, except for the white blood cell count (WBC). The highest vector dose (2.5×10^9 pfu) demonstrated an increased WBC count at Days 22 and 47, as well as some hypertrophy in the liver at Day 47, suggesting that this dose may have demonstrated toxicity that could not be detected at the lower doses. These changes in certain blood cell parameters and alterations to the liver could be expected for adenoviral inoculations combined with nucleotide analogues and should be part of the parameters that will be monitored in proposed human clinical trials.

VIII. INTRODUCTION

The sponsor is intending to treat metastatic prostate cancer with an adenovirus vector expressing the suicide gene, herpesvirus thymidine kinase (TK) gene followed by addition of the prodrug Valtrex (acyclovir). Prostate cancer in the United States is the leading male cancer diagnosis and the second most common cause of male cancer death. A large percent of all patients will have metastatic cancer at the time of diagnosis. The intended clinical trial will utilize *in vivo* inoculations to treat the solid tumors with an adenovirus vector delivering the TK gene under the control of the osteocalcin (OC) promoter, which can be targeted by the prodrug acyclovir. The bone specific promoter OC functions primarily in growing bone cells and in a series of tumors. Preliminary data has also indicated that when prostate cancer cells are infected, they can express high levels of TK activity in contrast to normal cells.

In order to proceed into the phase I clinical trials, this pre-clinical toxicology study examined three doses of vector delivered into the bones of the C57BL/6 mice. Vector was instilled into the right femur twice at Days 1 and 8, with Valtrex delivered daily from Day 2 through 21. Two control groups were utilized, one that received saline on Day 1 to the bone, and Valtrex daily for 21 days,

and another that received saline throughout all 21 days. This standard toxicology study was performed to examine general toxicity early after vector inoculation (Day 5), after the Valtrex was stopped at Day 22 and after a short recovery period (Day 47). At necropsy days samples were taken for blood cell counts, liver function tests, and tissue samples for histopathology.

IX. PROCEDURES

A. Test Material:

A.1   Description:

Recombinant virus, H5. OC-TK for these animals was supplied by the sponsor.

A.2   Lot:

1.9.98.1 Day 1 injections and 1.9.98.2 Day 8 injections.

A.3   Storage:

Stored at < -65°C to -80°C until use.

A.4   Expiration:

This was the responsibility of the Sponsor.

A.5   Handling:

All virus preparation was performed by University of Virginia personnel.

A.6   Control Article:

Sterile physiologic saline.

A.7   Identification of Test Material:

The University of Virginia personnel identified the virus to be used.

A.8   Archival Samples:

Unused test article was returned to the Sponsor for storage and analysis.

A.9   Test Article Analysis:

Unused test article was returned to the Sponsor for storage and analysis.

A.10   Unused Test Material:

This was returned to the Sponsor for storage and analysis.

B. TEST ANIMALS:

B.1 Genus/Species

Mouse (C57Bl/6); *Mus musculus*

B.2 Supplier:

Taconic

B.3 Justification for Test System Selection:

The study was designed to assess the general toxicity of varying doses of H5.OC-TK vector delivered via bone marrow injection in C57BL/6 male and female mice. This animal species has been used in a number of other studies to examine the toxicological effects of adenoviral inoculations. The University of Pennsylvania has used C57BL/6 mice in a number of studies to examine adenoviral inoculations through several routes.

B.4 Animal Requirements/Specifications:

a Number: 156

b. Age: 4-6 weeks

c. Sex: 78 Males and 78 Females

d. Weight: Approximately 18 - 25 gm.

B.5 Acclimation Period:

The mice were housed for a period of 2-8 days prior to experimentation.

B.6 Animal Husbandry:

a. Housing:

1 to 4 animals per shoebox cage in Thoren caging system supplied with HEPA filtered air.

b. Food:

Autoclavable Rodent Laboratory Diet # 5010 from PMI Feeds, Inc.

c. Water:

Acidified water was available from automatic watering system. The water supply is drawn from the City of Philadelphia. Copies of its analysis records are kept on file in the Animal Services Unit office, Institute for Human Gene Therapy.

· d. Bedding:

Hardwood Laboratory Bedding, coarse grade, Northwestern Products Corp., autoclaved prior to use

e. Veterinary Care:

Animals were monitored by the ASU staff and/or the Study Director and his staff for any conditions requiring possible intervention. The Study Director was consulted whenever possible. However, in emergency situations, decisions were made as needed and the Study Director was advised as soon as possible.

f. Environmental Conditions:

1) Light/Dark Cycle:

12 hour light/dark cycle (approximately 7 am to 7 pm) provided via automatic timer.

2) Temperature:

The temperature was to be maintained in a range of 64-79°F (18-26°C.).

3) Humidity:

Was not specified for this study.

B.7   Selection for Study:

Animals considered suitable for study on the basis of pretest physical examinations were randomly assigned to test group. Individual weights of animals placed on test were recorded (range from 13-22 grams). A small number of mice (9 of 156) did exceed the 20% of the mean body weight range, but all animals were randomly selected such that there was no bias to any group.

Each animal was assigned a permanent identification number by metal ear tag on the right ear. The number assigned by the test facility plus the study number comprised the unique animal number for each animal. Each cage was provided with a cage card, which included the study number, animal number, weight, treatment group or dose, sex, species, source, date received and Study Director.

C. Administration of Vector and GCV and General In-Life Procedure (See Table Below)

Experimental Design:

1. All mice were weighed at the beginning of the study and randomly assigned to the five groups and three sacrifice days using a permuted block randomization scheme with block sizes of 18.

2. Groups 1, 2, and 3 were inoculated with the H5.OC-TK vector at the doses of 2.5×10^7, 2.5×10^8, and 2.5×10^9 pfu/mouse, respectively, (see the schedule below for detail) through intramedullary injection on Day 1 into the right femur. On Day 1, the mice in Groups 4 and 5 received a single administration vehicle also intramedullary into the right femur. The surviving animals from all groups were injected again on Day 8 with the same dose and article as on Day 1.

3. The groups 1, 2, 3, and 4 were intraperitoneally (I.P.) injected with Valtrex daily from day 2 to 21 at a dose of 50 mg/kg in a volume of 0.05 mL sterile saline. The group 5 mice were inoculated with saline intraperitoneally (0.05 mL) daily from Day 2 to 21.

TABLE 1

Day	Treatment	Dose/Route	Proc/Samp	1*	2	3	4	5
1	H5.OC-TK	2.5×10^7 pfu in 25 ul		18/18	-	-	-	-
	H5.OC-TK	2.5×10^8 pfu in 25 ul		-	18/18	-	-	-
	H5.OC-TK	2.5×10^9 pfu in 25 ul		-	-	18/18	-	-
	Vehicle	25 ul saline		-	-	-	12/12	-
	Vehicle	25 ul saline		-	-	-	-	12/12
2-8	Valtrex	50 mg/kg I.P.		18/18	18/18	18/18	12/12	-
2-8	Saline	0.05 ml I.P.		-	-	-	-	12/12
5			Necropsy	6/6	6/6	6/6	4/4	4/4
			Clin Path	6/6	6/6	6/6	4/4	4/4
8	H5.OC-TK	2.5×10^7 pfu in 25 ul		12/12	-	-	-	-
	H5.OC-TK	2.5×10^8 pfu in 25 ul		-	12/12	-	-	-
	H5.OC-TK	2.5×10^9 pfu in 25 ul		-	-	12/12	-	-
	Vehicle	25 ul saline I.P.		-	-	-	8/8	-
	Vehicle	25 ul saline I.P.		-	-	-	-	8/8
8-21	Valtrex	50 mg/kg I.P.		12/12	12/12	12/12	8/8	-
8-21	Saline	0.05 ml I.P.		-	-	-	-	8/8
22			Partial Necropsy	6/6	6/6	6/6	4/4	4/4
			Clin Path	6/6	6/6	6/6	4/4	4/4
47			Full Necropsy	6/6	6/6	6/6	4/4	4/4
			Clin Path	6/6	6/6	6/6	4/4	4/4

*Numbers in each group refer to males/females respectively.

D. Observations

D.1 Viability Checks and General Observations:

The animals were observed daily for general appearance, signs of toxicity, distress and changes in behavior. Observations were performed by toxicology (veterinary) / TRP and ASU staff.

Animals found dead were evaluated, if possible, in the same manner as terminal sacrifice animals. A complete set of tissues was gathered and any gross lesions recorded.

D.2 Physical Examinations:

All animals were examined closely with daily checks and at the time of necropsy. Examinations included observations of general condition, surgical wounds, ocular/nasal discharges and general mobility. At the time of necropsy, the animals were examined for gross abnormalities.

D.3 Clinical Observations:

No clinical observations were noted.

D.4. Body Weights:

Animals were weighed at the beginning of the study, at necropsy, and every fourteen days (for those that were kept longer than 14 days).

E. Terminal Studies

E.1 Clinical Pathology

a. Hematology: Whole blood was collected at the time of necropsy into pre-labeled tubes and shipped to LabCorp for analyses. The table below lists the measurements made on the blood for various blood cell levels.

Table 2: Quantitative Hematology Parameters

Abbreviation	Parameter	Specimen	Method or Reference	Units
WBC	Total Leukocytes	Whole Blood	Abbott Cell Dyne 3500	x10³mm
RBC	Erythrocytes (Red blood cells)	Whole Blood	Abbott Cell Dyne 3500	x10⁶mm
HGB	Hemoglobin	Whole Blood	Abbott Cell Dyne 3500	g/dL
HCT	Hematocrit	Whole Blood	Abbott Cell Dyne 3500	%
MCV	Mean Corpuscular Volume	Calculated	Abbott Cell Dyne 3500	fl
MCH	Mean Corpuscular Hemoglobin	Calculated	Abbott Cell Dyne 3500	pg
MCHC	Mean Corpuscular Hemoglobin Concentration	Calculated	Abbott Cell Dyne 3500	g/dL
PLT	Platelets	Whole Blood	Peripheral blood smear	Visual estimate
POLY	Mature (Segmented) Neutrophils	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)
BAND	Band Neutrophils	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)
LYMPH	Lymphocytes	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)
MONO	Monocytes	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)
EOS	Eosinophils	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)
BASO	Basophils	Whole Blood	Manual Differential based upon counting 100 cells	% of WBC & Absolute Numbers (x10³)

b. Clinical Chemistry: Blood was also collected into pre-labeled red top tubes, allowed to clot and then centrifuged to obtain serum. The serum was removed and placed into pre-labeled tubes and sent to LabCorp for analyses. See Table 3 below for the liver enzymes and markers that were measured in this test.

The GGT assay is not valid in mice, as the values of zero are always obtained from LabCorp. However, LabCorp did include GGT results with the clinical chemistry evaluation. Therefore, no statistical evaluation or discussion of this parameter was included in this report.

Table 3

Abbreviation	Parameter	Specimen	Units
AST	Aspartate Aminotransferase	Serum	IU/L
T BILI	Total Bilirubin	Serum	mg/dL
GGT	Gamma-Glutamyl Transpeptidase	Serum	IU/L
ALT	Alanine Aminotransferase	Serum	IU/L
ALKPHOS	Alkaline Phosphatase	Serum	IU/L

E.2 Necropsy and Evaluation

a. Terminal Necropsy: Mice surviving to the end of their study period were euthanized via exsanguination under general anesthesia with ketamine (70-120 mg/kg) and xylazine (7-10 mg/kg) administered intramuscularly.

b. All Necropsies: Necropsies were performed on Days 5, 22 and 47 as per protocol (as amended). Necropsies were performed by the Veterinary Technicians (Lisa Stephens and Ernest Glover) with the observations being overseen by the Study Pathologist (Nelson A. Wivel, M.D.) and Study Director (Joseph V. Hughes, Ph.D.). The tissues taken are listed below under Histological Processing and Evaluation. Three areas were examined for enlarged lymph nodes (mesenteric, axillary and inguinal).

Tissues were placed in containers of Neutral Buffered Formalin (10%) and shipped to Experimental Pathology Corporation for preparation of slides and evaluation.

E.3 Histological Processing and Evaluation

Wet tissues were submitted to Experimental Pathology Laboratories, Inc., Research Triangle Park, North Carolina for preparation of hematoxylin and eosin stained microscopic slides and microscopic examination of tissues per the study protocol.

Hemotoxylin and eosin stained tissue sections were prepared for all protocol required tissues for all animals by the EPL histology laboratory as required by the study protocol. The following tissues were examined microscopically from all animals in all groups from both Day 5 and Day 22:

Bone, Sternum	Liver, Right
Bone, Femur	Lungs, Right
Brain, Cerebellum	Ovary, Right
Brain, Cerebrum	Prostrate
Cervix	Seminal Vesicle, Right
Gall Bladder	Spleen
Intestine-Large, Colon	Testes, Right
Intestine-Small, Duodenum	Thymus
Kidney, Right	Gross Lesions

On Day 47, the following additional tissues were also examined:

Esophagus
Heart
Stomach
Urinary Bladder

F. Statistical Analysis

All analyses were performed using the SAS (1) system and all plots were created using the S-plus statistical analysis package (2). All Clinical Pathology Laboratory parameters were examined including blood cell counts, and liver function test. Body weights were analyzed for values taken at the time of the first vector injection and at the time of sacrifice. The comparison of injection weights provided some evaluation of the comparability of the randomized treatment groups.

The design of this study allowed for the examination of group effects, overall time effects (day of necropsy) and group by day interactions using ANCOVA (analysis of covariance) while also examining a factor for gender. In the primary analysis, the Groups 1, 2, 3, and 4 were compared using the actual dose level of the vector as a continuous covariate, with Group 4 equal to 0. The control groups (4 and 5) were also compared as a categorical variable. Both analyses included factors for day and gender and examined group by day interactions. When group interactions were not significant (p > 0.05) the interaction term was dropped from the model and only the main effects were fit. Analyses were also reviewed within gender.

X. RESULTS SECTION

A. Observations:

A.1 Mortality

There were ten animals that died during this study. Primarily these deaths occurred on the days of vector instillation. On Day 1, four mice died (one in Group 1, one in Group 2, and two in Group 4); these were apparently the results of the anesthesia and the surgical procedure. Again at the second instillation on Day 8, three animals (One group 3 and two Group 5) were lost during the procedure. Two (One Group 3 and one Group 4) also died on Day 9 within 24 hours of the second vector administration. In general, these were in both the vector and control groups and were most likely related to the procedure rather than material inoculated. One mouse in Control Group 5 was found dead on the last day of study, Day 47; no specific results could be detected upon necropsy of this animal.

A.2 Clinical Signs

No clinical signs were noted through Day 47.

A.3 Body Weight

All five groups gained weight throughout the study (data in Appendix C, analysis in Appendix D). There were no differences detected between groups in the weights or weight gain from the Day 1 of the first vector instillation to the Day of necropsy for any of the three dose groups or the two control groups.

B.   Terminal Studies

B.1 Clinical Pathology

The data for the blood cell counts and liver function tests are contained in Appendix C. This data was subjected to a statistical analysis, which is contained in Appendix D. A summary is provided in the text below.

a. Hematology

The primary analysis was a dose response analysis of covariance of all groups which received Valtrex. The statistical model included factors for day, dose, gender and dose by day interaction (Table 4). Only p-values less than 0.05 are shown; the rest are denoted as Not Significant (NS).

No statistically significant interaction was found between dose and day of sacrifice for any parameters.

The white blood cells (WBC) did demonstrate a statistically significant dose effect. Primarily this appears to be due to the high vector dose group which demonstrated an increased WBC cell count, particularly at Days 22 and 47. The two lower doses of vector appeared more similar to the control (saline) Group 4 values for the WBC counts. The eosinophils demonstrated only a mildly statistically significant effect for the dose, which is apparently due to slightly decreased values for the eosinophils primarily at Days 22 and 47 of the study. None of the other blood cell levels demonstrated significant changes that were attributable to the vector dose instilled into these animals.

There were a number of blood cell parameters which changed over time, but these changes happened to all groups (vector or saline control). The following parameters demonstrated a significant time effect.

1. WBC which showed an increase.

2. MCV, MCH, MCHC, and Hematocrit values which declined over time within the study.

3. Eosinophils reflected a small decrease in terms of absolute numbers.

A second set of statistical analyses examined the effects of Valtrex, by comparing Groups 4 and 5 (with Valtrex administration or vehicle alone, respectively). In this analysis, there were no significant differences between the two groups related to blood cell levels; similar differences were detected over time with changes in WBC, MCV, and MCHC. (See analyses in Appendix D)

Table 4: Hematology and Liver Function Tests
ANCOVA Models Examining Dose, Day and Dose by Day Interaction, Controlling for Gender – Groups 1, 2, 3, and 4.

Outcome	Dose	Day	Dose x Day	Gender
WBC	0.0001	0.0001	NS	0.012
RBC	NS	NS	NS	NS
Hemoglobin	NS	NS	NS	NS
Hematocrit	NS	0.033	NS	NS
MCV	NS	0.0001	NS	NS
MCH	NS	0.024	NS	0.002
MCHC	NS	0.0001	NS	NS
Polycytes	NS	NS	NS	NS
Lymphocytes	NS	NS	NS	NS
Monocytes	NS	0.050	NS	NS
Eosinophils	0.030	0.0001	NS	NS
AST (SGOT)	NS	0.035	NS	0.015
ALT (SGPT)	NS	NS	NS	NS
Alk Phos	NS	0.0001	NS	0.0001

b. Liver Function Tests (LFT's)

The values of AST and ALT appeared elevated at the Day 5 necropsies for all groups. The LFTs dropped considerably by Day 22 and 47. The primary analysis (Table 4) comparing the vector dose groups (1 through 4); did not demonstrate any LFT value that was affected by the dose of the vector.

Some significant effects were detected over time (Table 4) particularly for AST and Alkaline Phosphatase, which both demonstrated an increase. However, the overall values are similar between the groups receiving vector and the saline control group.

B.2   Necropsy: Gross Pathology

The results of gross pathology reviewed from the necrospy is summarized in Table 5. At Day 5, a number of animals were found to have hematomas on the right thigh muscle. This was found in the saline control groups as well as the vector groups and could be due to vector treatment or to the intramuscular injection of anesthetic. Day 47 lesions also included injection site focal areas that appeared to be recovering; these were present only in females.

In addition, one animal (Group 3 high dose vector) demonstrated a darkened tip of the spleen 5 days after vector instillation. At Day 22, a number of the female mice demonstrated an enlarged ovary and fallopian tube.

Table 5: Gross Pathology at Necropsy

		Groups (Males/Females)				
		1	2	3	4	5
Day 5	(1) Hematoma, Right Thigh Muscle	1/0	1/1	0/0	3/0	3/2
	(2) Spleen, Darkened Tip	0/0	0/0	1/0	0/0	0/0
Day 22	Ovary and Fallopian Tubes, Enlarged	NA/1	NA/2	NA/2	NA/1	NA/0
Day 47	Injection Site, Focal Hemorrhage	0/1	0/2	0/3	0/0	0/2

B.3   Histological Findings

Tissue samples submitted to EPL on Days 22 and 47 had evidence of autolysis present when examined by EPL. Almost none of the study tissues for Day 22 could be evaluated for toxicity as a result of the autolysis (almost no nuclear detail left in any slide). The cause of the autolysis was not certain, but improper fixation was the likely cause.

The remainder of the tissues were examined and the results are detailed in Appendix E.

Femur

A number of changes were present in the femur of mice at all time points. The results for both sexes on Day 5 are shown in Table 6.

B.3a   Histopathology of femur – Day 5

A number of histopathological changes were noted in the femur, most prominently in the males (Table 6). None of the patterns was consistent across groups, except for traumatic necrosis seen in all groups, but Group 4 males. No clear pattern relating to either vector or Valtrex was noted.

Table 6 – Femur: Mean Scores per Animal – Day 5

Group	1	2	3	4	5
Males					
Number Examined	(6)	(6)	(6)	(4)	(4)
Fibrosis, Articular, Diffuse	1 (0.2)	0	0	0	0
Fibrosis, Articular, Focal	0	0	0	2 (0.8)	0
Fibrosis, Periosteal, Focal	0	1 (0.2)	0	0	1 (0.5)
Inflammation, Synovial, Acute, Diffuse	1 (0.3)	0	0	1 (0.5)	0
Inflammation, Synovial, Acute, Focal	1 (0.3)	0	0	2 (0.5)	1 (0.3)
Traumatic Necrosis	1 (0.3)	2 (0.7)	5 (1.3)	0	1 (0.3)
Females					
Number Examined	(6)	(6)	(6)	(4)	(4)
Fibrosis, Periosteal, Focal	0	1 (0.3)	0	0	0
Inflammation, Synovial, Acute, Focal	2 (0.5)	0	0	0	0
Traumatic Necrosis	1 (0.3)	5 (2.0)	5 (2.0)	4 (2.5)	4 (2.0)

B.3b   Histopathology of femur - Day 22

On Day 22, only a small number of the femurs in female mice could be evaluated, due to the autolysis present in almost all tissues in these mice. Fibrosis was present in one mouse in Group 4 and Group 3. Traumatic necrosis was present in three of four mice in Group 5 and one of six mice in Group 3. The results are shown in the following table:

Table 7 – Femur: Mean Scores per Animal – Day 22

Group	1	2	3	4	5
Females					
Number Examined	(6)	(6)	(6)	(4)	(4)
Fibrosis, Articular, Focal	0	0	0	1 (0.8)	0 --
Fibrosis, Periosteal, Focal	0	0	1 (0.3)	0	0
Traumatic Necrosis	0	0	1 (0.3)	0	3 (1.0)

B.3c   Histopathology of femur - Day 47

By Day 47, the character of the lesions in the femur had changed. Fibrosis, acute inflammation and traumatic necrosis were no longer present, having been replaced by a group of more chronic lesions. These lesions included foreign body reaction which consisted of small clusters of foreign body giant cells which surrounded lipid deposits in the marrow cavity; and disruption of the epiphyseal plate, which was graded as present when there was a frank break in the epiphyseal plate; and remodeling of epiphysis, which consisted of a distortion of the shape of the end of the femur as a result of the disruption of

the epiphyseal plate. No clear relationship to dose of vector or Valtrex was noted on Day 47. The incidence of these lesions at Day 47 is shown in the following table:

Table 8 - Femur, Mean Scores per Animal – Day 47

Group	1	2	3	4	5
Males					
Number Examined	(6)	(6)	(6)	(3)	(4)
Disruption, Epiphyseal Plate	5	2	2	1	1
Foreign Body Reaction	5 (1.2)	3 (0.8)	2 (0.3)	2 (1.0)	4 (1.3)
Females					
Number Examined	(6)	(6)	(6)	(4)	(4)
Disruption, Epiphyseal Plate	3	4	4	3	3
Foreign Body Reaction	3 (0.7)	4 (1.3)	6 (1.5)	3 (1.8)	3 (1.3)

Lung

As a replicating adenovirus may affect the lungs, the lungs were examined by using the criteria described by Ginsberg et al. 1991. The following lesions were recorded in the lungs: Alveolar Infiltration, Peribronchial Infiltration and Perivascular Infiltration. The incidence of these lesions was extremely low for all groups at all days and consistent across groups.

Liver

Extramedullary hematopoiesis and focal mononuclear cell infiltration was present in the liver at all time points, with no apparent relationship to treatment with H5-OC-TK vector. On Day 47, several changes characterized as hypertrophy were present in the livers of Group 3 mice that had received the highest vector dose. All other groups had no evidence of this histopathology.

Other Organs

No other consistent alterations were noted in any tissues examined at any timepoints. The results are listed in Appendix E.

XI.   CONCLUSIONS AND DISCUSSIONS

No clinical symptoms were observed in daily observations during the in-life phase of this study for any of the doses of vector or the control group. The body weights for the C57BL/6 mice increased at similar rates for all groups independent of vector concentration or Valtrex inoculation. Most of the toxicity noted in this study was apparently a result of the method of instillation rather than the vector or the prodrug treatment with Valtrex. While there were some deaths on study, these occurred primarily on the days of vector instillation or within the first 24 hours (on either Day 1 or 8). The deaths occurred in both vector and Valtrex control animals, and were apparently related to the surgical instillation and not the vector or Valtrex. One mouse in the control Group 5

(saline injections only) was found dead on the last day of the study. No specific results were determined from the necropsy of this animal.

The results from the blood cell counts and analyses of liver function tests also demonstrated there was no statistical difference for most of measurements between the various doses of the vector and the Valtrex treatment. The white blood cells counts (WBC) was the only exception. There was increased WBC counts for the groups which received the highest dose of vector. The absolute number change is not very high and the highest dose of vector was clearly very different from the control (no vector group) and the low and middle dose groups. It is possible there was a combined effect of the Valtrex and vector. We have seen in other studies that similar combinations of TK enzyme and nucleotide analogues affect some blood cell counts, including the WBC. Clearly the WBC count could represent a parameter that should be carefully monitored in the proposed clinical trial, but this should receive close scrutiny in the dose escalation trial that is proposed.

Almost none of the study tissues for Day 22 could be evaluated histologically for toxicity as a result of the autolysis (almost no nuclear detail left in any slide). The cause of the autolysis was not certain, but improper fixation was the likely cause. The gross pathology and microscopic alterations observed with the histopathology of the tissues taken at necropsy were primarily the result of the process of instillation. The most prevalent changes consisted of hematoma (early time point) and hemorrhage (at Day 47) which were found consistently throughout the control and vector groups, suggesting these changes were independent of vector or Valtrex. The enlarged fallopian tubes and ovaries were not easily examined histologically because of the autolysis of some of these tissues. The one set of tissues examined did not reveal any underlining pathological changes. This change was seen in all groups that received Valtrex, and was not seen at any other time points. This particular observation should not be a significant factor in the proposed clinical phase 1 trial for metastatic prostate cancer, although care should be taken during the clinical trials as the effect is not well understood.

The histopathologic lesions present in the femur were considered to be the result of the experimental manipulation (injection into the marrow cavity using a needle), rather than a result of the vector itself. These lesions were present in both vector and vehicle groups, and changed from acute to chronic during the course of the study.

The histopathologic lesions in the liver in Group 3 (2.5x10^{9}pfu) on Day 47 are of the type that have previously been seen in the liver as the result of adenoviral vector administration to C57BL/6 mice. LFT data, however, suggests there was no significant hepatotoxicity occurring, as the ALT and AST values were similar for all vector and control groups. The histopathologic changes in the lung were not considered to be significant. There were no changes in any of the abdominal organs examined that were considered the result of the repeated intraperitoneal injections of Valtrex.

Other histopathological changes were centered on changes in the femur site of the vector or saline control instillation. The changes were transient progressing from fibrosis, inflammation and traumatic necrosis to more chronic lesions including evidence of foreign bodies and disruption of the epiphyseal plate. These histological changes were present throughout the groups, independent of vector or Valtrex instillation and therefore most likely related to the procedure and not either the vector or Valtrex. The highest vector dose group (Group 3, 2.5x10^9 pfu) demonstrated some hypertrophy in the liver at Day 47, which is consistent with previous adenoviral vector induced changes.

The changes in the liver and the increased WBC count to the highest vector dose suggests that this dose could be approaching a toxic endpoint for this vector and drug combination. However, there were no severe clinical signs, deaths, body weight changes, nor other LFT alterations. It should expected that changes in the liver and some blood cell counts could be affected by adenovirus vector expressing the TK enzyme in combination with acyclovir. These parameters should be carefully monitored in any proposed clinical trial.

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Novel Prostate-Specific Promoter Derived from PSA and PSMA Enhancers

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The expression of prostate-specific membrane antigen (PSMA) and prostate-specific antigen (PSA), two well characterized marker proteins, remains highly active in the hormone refractory stage of prostate cancer. In this study, an artificial chimeric enhancer (PSES) composed of two modified regulatory elements controlling the expression of PSA and PSMA genes was tested for its promoter activity and tissue specificity using the reporter system. As a result, this novel PSES promoter remained silent in PSA- and PSMA-negative prostate and non-prostate cancer cell lines, but mediated high levels of luciferase in PSA- and PSMA-expressing prostate cancer cell lines in the presence and absence of androgen. To determine whether PSES could be used for in vivo gene therapy of prostate cancer, a recombinant adenovirus, Ad-PSES-luc, was constructed. Luciferase activity in prostate cancer cell lines mediated by Ad-PSES-luc was 400- to 1000-fold higher than in several other non-prostate cell lines, suggesting the high tissue-specificity of the PSES promoter in an adenoviral vector. Finally, recombinant virus Ad-PSES-luc was injected into mice to evaluate the tissue-discriminatory promoter activity in an experimental animal. Unlike Ad-CMV-luc, the luciferase activity from systemic injection of Ad-PSES-luc was fairly low in all major organs. However, when injected into prostate, Ad-PSES-luc drove high luciferase activity almost exclusively in prostate and not in other tissues. Our results demonstrated the potential use of PSES for the treatment of androgen-independent prostate cancer patients.

Key Words: PSA, PSMA, prostate, gene therapy, adenovirus

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men annually [1]. Initially, prostate cancer development is androgen-dependent. However, it evolves to an androgen-independent stage by unknown molecular mechanisms and becomes refractory to hormone ablation therapy. Currently, there are no effective therapeutic agents available for patients with hormone-refractory prostate cancer.

Among several prostate-specific proteins, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are sensitive markers for prostate cancer diagnosis and progression. PSA is a serine protease produced by the prostatic ductal epithelial cells and secreted into the seminal plasma. The basement membrane acts as a barrier to block PSA from escaping into systemic circulation. Prostate cancer or benign hyperplasia disrupts the membrane, eliciting significant increases in serum PSA. Therefore, serum PSA is used as a molecular marker for prostate abnormalities.

PSA expression is modulated by androgens, acting through androgen receptor (AR) like other nuclear receptor families [2]. Upon binding androgen, primed AR is translocated into the nucleus and binds the proximal promoter and enhancer core region (AREc) 4.2 kb upstream of the PSA gene [3–5]. The PSA regulatory element or its modification exhibits strong tissue-specific activity and has been used to drive therapeutic gene expression targeting prostate cancer [6,7]. The transcriptional levels mediated by PSA promoters are, however, significantly lower in the absence of androgen, which potentially limits its application in patients under androgen ablation therapy. Transcription levels must be improved for effective therapeutic results; a highly active promoter with stringent tissue-specificity is crucial for delivering foreign DNA to target tissue.

PSMA was discovered as a membrane antigen reactive to monoclonal antibody 7E11-C5 [8]. The physiological role of PSMA in prostate remains unknown. It has hydrolase

A

AATTA<u>TTTTTTCCTTT</u>AACCTTTCAAACTCAAGGAAAACCAGTTGG
<u>CCTTGACTCTGT</u>TTGTGGAAAATTTTAAACTACTGGTTTAATTT
CTTTATTGGTTGTAA<u>TATGACTAT</u>TTTACGTCATATAACAATTTT
TATTGTTTGTTAA<u>ATGACTTT</u>ATTGT<u>TTGTCATATGATAAT</u>TTTAT
GTCATAGAACAATTTTTATTGCTTGATA<u>TATGACTTT</u>ATTGTTATA
TGGCTATACAACTAGAT<u>TTTTTT</u>GTTGTTT<u>TTT</u>gaccgagtcttactctgtc
acccaggctggagtgtaatggcatggtctcagctcactgcaacctccgcctcccggg

FIG. 2. Deletion analysis of PSME. (A) Sequence of PSME located in the third intron in the PSMA gene (FOLH1). PSME contains nine AP-1 sites, a repeat sequence (bold), and an Alu sequence (lower case). (B) Left panel schematically illustrates the deletions of PSME used in this study. The shortened versions of PSME were inserted upstream of TATA in pGL3/TATA. The transcriptional activ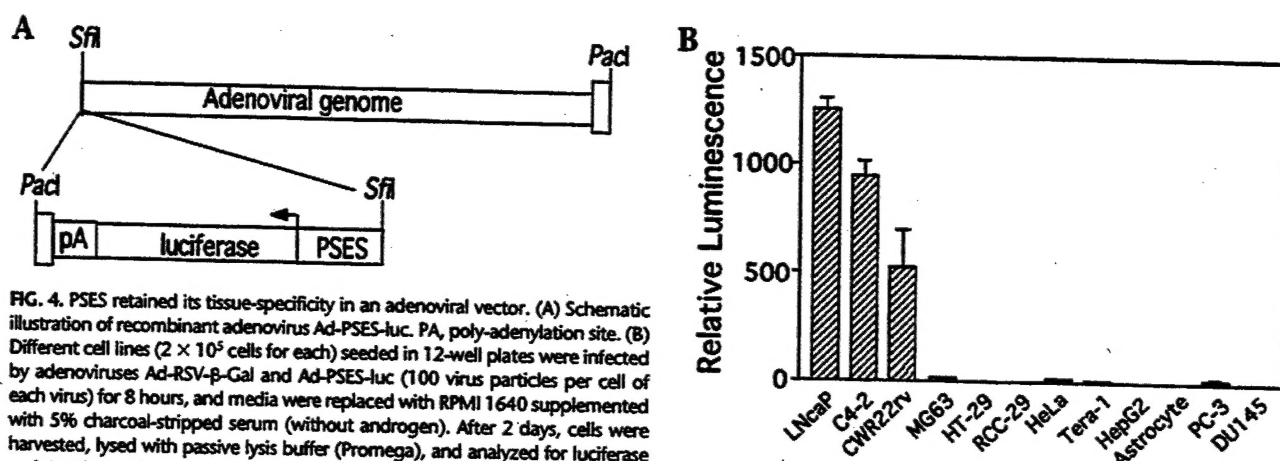ities of these constructs were then evaluated by transient reporter assay as described in Fig. 1. Results are presented as mean ± SD.

B

three AR binding sites and six GATA transcription factor binding sites, suggesting that GATA is involved in the optimal androgen induction.

PSME, which is within the third intron of the PSMA-encoding gene, enables the prostate-specific gene expression of PSMA in the absence of androgen [14]. We constructed a series of deletions to further locate prostate-specific enhancers within PSME (Fig. 2A) and evaluated their enhancing activities. PSME (del2), which lost the Alu-repeat sequence (262 to 327 bp) located at the end of PSME, had four- to fivefold higher activity than PSME, suggesting that the Alu repeat contains a suppressive regulatory element (Fig. 2B). The Alu repeat belongs to the SINE (short interspersed element) family of human repetitive sequences [17]. Although the function of Alu-repeat sequences is not well understood, several studies have identified transcriptional silencers within Alu-repeat sequences and suggested that Alu-repeat sequences might be involved in transcriptional regulation [17]. This study provides additional data suggesting a role for Alu-repeat sequences in gene regulation.

In contrast to PSME (del2), PSME (del3), which lacks bp 1–90, had much lower promoter activity compared with the full-length PSME, indicating that this region upstream of direct repeat sequences (Fig. 2A) harbors one or more enhancer elements. According to sequence analysis, this upstream region of PSME, bp 1–90, includes an activator protein 1 (AP-1) binding site and three AP-3 binding sites (Fig. 2A). These potential regulators may be involved in the transcriptional activation of PSMA gene expression.

Mutant PSME (del4), which lacks both the 90-bp upstream enhancer and the Alu repeat silencer (Fig. 2B), had activity that was moderately higher than that of PSME (del3) but lower than that of PSME (del2), reflecting the overall level of promoter activity without silencing or enhancing regulatory elements in the regions of deletion of bp 1–90 and bp 262–327, respectively.

All PSME deletions shown in Fig. 2B showed downregulation in the presence of androgen, suggesting that the regulatory element responsible for androgen-mediated downregulation of PSME activity resides in the direct repeat sequence (Fig. 2A). Interestingly, AR was demonstrated to inhibit c-Jun/AP-1 site interaction by forming a complex with c-Jun without directly binding to the AP-1 site [18]. Because there are nine AP-1 sites within and upstream of the direct repeat sequence, AP-1s may act as positive regulators and their activities may be downregulated by androgen.

PSA promoter, which has been investigated for prostate cancer gene therapy [6,7], is a tissue-specific promoter whose activity heavily depends on androgens. We reported that PSA promoter had higher activity in androgen-independent C4-2 cells than in androgen-dependent LNCaP cells in the absence of androgen [16]. However, its activity in C4-2 cells in the absence of androgen is still much weaker than that of the commonly used simian virus 40 (SV40) or Rous sarcoma virus (RSV) promoters. This lower level of promoter activity potentially hampers its application in patients for androgen ablation therapy. On the other hand, the enhancer activity of PSME (del2) was higher in the absence of androgen but still significantly lower compared with constitutively active viral promoters. We believed that both AREc3 and PSME (del2) were likely to function weakly in patients under androgen ablation therapy due to the patients' low levels of androgen and to AR mutation or amplification, which resulted in partial activation of AREc3 and suppression of PSME (del2) activity (unpublished data). We hypothesized that the combination of AREc3 and PSME (del2) might have a synergistic enhancer effect, balance out the positive and negative regulatory effect of androgen, and retain tissue specificity.

A

B

FIG. 4. PSES retained its tissue-specificity in an adenoviral vector. (A) Schematic illustration of recombinant adenovirus Ad-PSES-luc. PA, poly-adenylation site. (B) Different cell lines (2 × 10⁵ cells for each) seeded in 12-well plates were infected by adenoviruses Ad-RSV-β-Gal and Ad-PSES-luc (100 virus particles per cell of each virus) for 8 hours, and media were replaced with RPMI 1640 supplemented with 5% charcoal-stripped serum (without androgen). After 2 days, cells were harvested, lysed with passive lysis buffer (Promega), and analyzed for luciferase and β-galactosidase activities. The luciferase activities are normalized with β-galactosidase activity. Results are presented as mean ± SD.

carrying the luciferase gene under the control of the cytomegalovirus (CMV) promoter, or Ad-PSES-luc. After 3 days, we measured the luciferase expression in different mouse organs. High levels of luciferase activity were detected predominantly in liver, spleen, and lung, but levels were negligible in other tissues obtained from mice injected with Ad-CMV-luc(Fig. 5A). Unlike Ad-CMV-luc, Ad-PSES-luc was inactive in all organs tested. However, when injected directly into prostate (Fig. 5B), Ad-PSES-luc was highly active in prostate. These data demonstrated that PSES in the form of Ad-PSES-luc was active in normal mouse prostate with high tissue specificity.

In this study, we developed a novel prostate-specific chimeric enhancer, PSES, from the enhancer cores of PSA promoter, AREc3, and PSME (del2). This PSES was highly active in PSA- and PSMA-positive prostate cancer cells in the presence and absence of androgens, exhibiting strong tissue-specificity as an adenoviral vector. Its strong androgen-independent promoter activity makes PSES superior to PSA and PSMA promoters for the treatment of patients undergoing androgen ablation therapy.

We are currently investigating the use of PSES to control the replication-competent oncolytic adenovirus for the treatment of androgen-independent prostate cancer. Although PSA enhancer and promoter were used to drive the prostate-specific replication of oncolytic adenoviruses [20], we believe that PSES-based replication-competent adenovirus would be more efficacious for the treatment of androgen-independent prostate cancer.

MATERIALS AND METHODS

Cells and cell culture. LNCaP, C4-2, CWR22rv, PC-3, and DU145 prostate cancer cell lines were maintained in T medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). C4-2 was purchased from Urocore (Oklahoma City, OK). CWR22rv was obtained from Liang Cheng (Indiana University). LNCaP was obtained from Leland Chung (Emory University). Human liver carcinoma HepG2, human cervix cancer cell line HeLa, human testicular cancer cell line Tera-1, human kidney cancer cell line RCC-29, human colon cancer cell line HT-29, human osteoblast-derived osteosarcoma cell line MG63, and human prostate cancer cell lines PC-3 and DU145 were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS and 1% P/S.

FIG. 5. PSES promoter in the form of Ad-PSES-luc remains specifically active in the prostate of *in vivo* mice. (A) We injected 7 × 10¹⁰ virus particles of recombinant virus Ad-CMV-luc (filled bar) or Ad-PSES-luc (open bar) into tail veins of male athymic mice. After 2 days, organs were harvested from sacrificed mice and homogenized. Protein extracts (0.48 mg) were used to determine luciferase activities. (B) We injected 1.4 × 10¹⁰ virus particles of recombinant virus Ad-CMV-luc (filled bar) or Ad-PSES-luc (open bar) into prostates of male athymic mice. Protein extracts were obtained and used as described for luciferase activities.

A

Tail vein injection

B

Intra-prostate injection
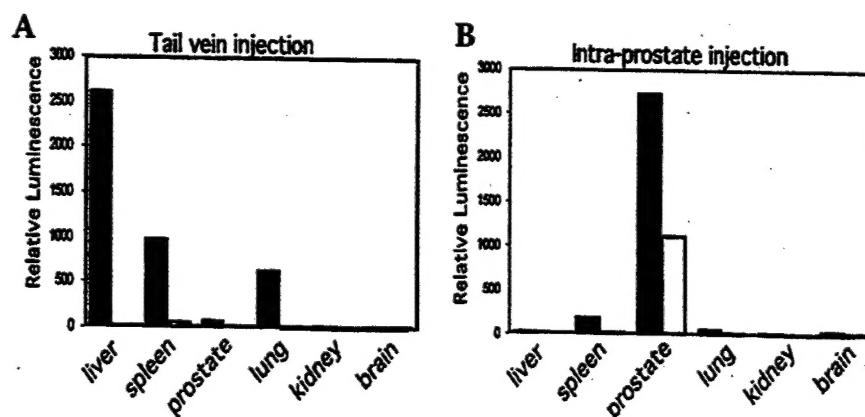
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